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GENETIC BASIS OF A SPECIFIC PHENOCOPY RESPONSE

by

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ABSTRACT

GENETIC BASIS OF A SPECIFIC PHENOCOPY RESPONSE

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Utilizing glucosamine phenocopy susceptibility as an indicator of developmental homeostasis of the aminosugar pathway in D. melanogaster experiments were performed to explore the genetic nature of this buffering.

Homozygous second and third chromosomes were derived from four laboratory strains shown to have different phenocopy susceptibilities. Experiments using the Latin-square design were performed to determine if 1) a laboratory stock is genetically homogeneous with respect to phenocopy susceptibility; 2) the genes responsible for phenocopy susceptibility act in an additive, dominant or recessive, or epistatic manner; and 3) if phenocopy susceptible genes in chromosomes derived from different strains are allelic.

There was no detectable genetic polymorphism for phenocopy susceptibility in the strain tested (e¹¹). The implications of this finding for considering phenocopy susceptibility as a threshold phenomenon were discussed. Dominant alleles were shown to confer homeostatic properties on the organism and the data are consistent with the view that different inbred strains use different alleles to achieve homeostasis. Interactions were found to play some part in the genetic con-

trol of phenocopy susceptibility. For example, while heterozygosity was generally associated with phenocopy tolerance, single chromosome heterozygotes were found in some cases to be more tolerant than double heterozygotes. These results were considered in relation to gene balance phenomena and their implications for developmental homeostasis.

These data were also discussed in terms of the control of metabolic patterns. The study indicates that the utilization of excess endogenous metabolites to test for the existence of genes controlling developmental buffering may provide a useful method of approaching problems of developmental homeostasis.

To my mother and the memory of my father

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INTRODUCTION

There is a large body of evidence (Dobzhansky, 1951; Lerner, 1954) which indicates that members of a species developing with different genetic and environmental backgrounds can emerge as morphologically indistinguishable adults. On the basis of such findings it has been suggested (Schmalhausen, 1949) that the development of an organism toward the "normal phenotype" is buffered in order to resist fluctuations in the environment. This buffering process has been called developmental homeostasis or canalization of development (Dobzhansky and Wallace, 1953; Lewontin, 1956, 1957).

Although the problem of genetic control of developmental homeostasis has long been recognized, only two promising experimental approaches to the analysis of this problem have emerged. One approach utilizes selection programs for occasional phenodeviants. After several generations of selection, populations with a high frequency of the selected characteristic are obtained. From such factors as the number of generations of selection required to achieve high frequencies inferences are made concerning the nature of the genes controlling the stability of a given phenotype (Rendel, 1967). This approach has several drawbacks. Such experiments routinely take fifty or more generations and in themselves reveal little about the genetic architecture of the

trait. Furthermore, the quantitative morphological traits usually used, such as bristle number in Drosophila, are probably the result of the interaction of several complex developmental pathways which are too complicated to permit ready analysis of specific biochemical pathways.

Another approach consists of studying the effect of phenocopy agents. A phenocopy is like a mutant phenotype, and is produced by treating an organism with a physical or chemical agent. The original phenocopy experiments utilized temperature shocks as the inducing agent. Work was done on wing pattern phenocopies in butterflies and moths, on phenocopies of diverse mutants in Drosophila and on skeletal phenocopies in fowl. A review of the early phase of phenocopy studies is presented in Goldschmidt (1938). The first studies utilizing chemical phenocopy agents in Drosophila were reported by Rapoport (1949) who showed that mercury caused a phenocopy of the Minute-type while silver salts caused yellow or straw phenocopies. Antimony produced a brown-like phenotype whereas boron produced several of the characteristics associated with eyeless. The chubby phenotype was produced by thiocyanate compounds and trichloroacetic acid was associated with the phenocopy chrome. These studies were followed by those of Sang and MacDonald (1954) who demonstrated that the phenocopy agent sodium metaborate could change the threshold for the expression of the eyeless (ey) gene. Their results showed that the +/+ genotype was

less affected by the salt than was ey/+ which showed a greater tolerance to sodium metaborate than did ey/ey. Goldschmidt and Piternick (1957 a, b) also using borates, have greatly extended these investigations. One of the important results of such studies is that laboratory strains respond to phenocopy agents with a frequency characteristic of the strain. Landauer (1957) has documented this finding in poultry as well. Thus the ability to respond to a phenocopic agent appears to be under genetic control.

Despite agreement on this point, there is no agreement as to the interpretation of the relationship of genes to the phenocopic response. Landauer contends that a high phenocopy response indicates that the modifier genes which buffer particular developmental pathways are ineffective while Goldschmidt argues that phenocopy agents reveal the presence of a previously concealed mutant gene or genes. The agent acts to change conditions so that mutants with sub-threshold action can express themselves (Goldschmidt, 1957a, b). The interpretations are difficult to distinguish between since they are not mutually exclusive. For present purposes it suffices that either interpretation is consistent with the premise that phenocopy phenomena offer a tool for investigating the manner in which the developing organism copes with changing internal and external environments.

In order to be useful a phenocopy system should fulfill certain requirements. First, the phenocopic agent should interfere with a specific

developmental pathway and result in a homogeneous response. Secondly, the agent must have a quantitatively differential effect on different strains so that genetic control of the response can be examined. Such a system might allow the genetic aspects to be elucidated and perhaps ultimately related to the cellular and biochemical events responsible for the altered phenotype.

A phenocopic agent which fulfills these criteria was discovered by Rizki (1960). He found that an endogenous metabolite, glucosamine, when fed to Drosophila melanogaster larvae caused the production of a phenocopy of the cryptocephal (crc) gene. The crc phenotype, first described by Hadorn and Gloor (1943) is characterized by the inability of the pupa to evert its head. The effect is conspicuous by virtue of the fact that both pigmented eyes remain within the thorax, and the larval mouth parts are embedded in pupal tissue. The syndrome also includes shortened wings and legs. Needless to say the condition is lethal. The glucosamine phenocopy faithfully mimics the morphological characteristics of the crc syndrome and does not seem to affect any other characters. This contrasts with other agents, chemical and physical, which have caused a heterogeneous response. Gloor treated pre-pupae with high and low temperature shocks (1944) and ether (1947) producing the crc phenocopy among others. Epstein obtained similar results utilizing ultra-violet light. These agents apparently produce the effects associated with them by arresting developmental processes at particu-

lar stages. For this reason they are not particularly useful for the study of the homeostatic properties of specific pathways. Further evidence for the non-specificity of previously described phenocopy agents can be found in the studies of Goldschmidt and Piternick (1957 b, c). Returning to properties of the glucosamine caused phenocopy, Rizki (1960) also showed that the Ore-R strain was phenocopied less frequently than was the tu^W strain; thus the genetic control of the phenocopy response was indicated. Fristrom (1964, 1965) has shown that glucosamine administration upsets a specific biochemical pathway in larvae of Drosophila. Theoretically, in any given metabolic pathway many genes are involved, hence the response to glucosamine feeding could be utilized to study genetic regulation of the pathway. Tolerance of a given stock to ingested glucosamine could be considered a measure of genetically permissive developmental homeostasis with respect to glucosamine metabolism.

Several types of information are necessary for partitioning the influence of various genetic elements which control this system. First of all the meaning of the frequency of phenocopy response must be established. Either the frequency represents the realization of the ability for any individual in the population being susceptible or it indicates that the population is genetically polymorphic and that only a certain percentage of the zygotes in a population are endowed with respon-

sive genotypes. If the latter situation exists then it would be necessary to synthesize genetically homogeneous strains in order that genetic elements influencing the system can be identified. Furthermore, as there is no assurance that any strain will be uniform since mutations accumulate with time, the development of isogenic lines may be deemed necessary for performing a genetic analysis of the system. Once strain homogeneity has been established, the types of gene action involved in the phenocopy response may be revealed through an analysis of the phenocopy responsiveness of appropriate genotypes. Experiments can be designed to answer the following questions which bear on the genetics involved in stabilization of developmental processes.

a) If we consider the genes as the determining factors of the responsive system then what is the distribution of these determinants on the first, second, and third linkage groups of D. melanogaster? The clustering of such genes in a particular linkage group might suggest a functional interdependence among loci.

b) Do the genetic determinants of glucosamine phenocopy susceptibility act in an additive, dominant or epistatic manner?

c) Are the genes responsible for phenocopy susceptibility on chromosomes from different strains, allelic? Answers to the latter questions should provide clues to the possible ways in which genes might control the pathways involved in the phenocopy response.

METHODS AND MATERIALS

Stock cultures of Drosophila melanogaster were maintained on a medium of cornmeal and agar, enriched with a drop of live yeast solution prior to inoculation by mass transfer. Stocks were generally maintained at $25^{\circ}\text{C} \pm 1^{\circ}$. In addition, a set of duplicate stocks was maintained at 15°C , beginning in August, 1967.

The mating scheme for preparing stocks homozygous for particular second and third chromosomes is presented below. The technique requires that the derived chromosomes be balanced by dominant markers which are homozygous lethals and which are associated with inversions used to suppress crossing over. The desired chromosome can then be obtained intact in the homozygous state during subsequent generations (Muller and Oster, 1963). The balanced lethal chromosome stock used was the j172 stock obtained from Dr. I.I. Oster. This stock has the genetic constitution $S\ Sp\ cn/dp^{tx^I}\ Cy, Ins\ O\ pr\ cn^2; h\ ri\ D^3\ InC\ e\ l3e/Me, Ins\ ri\ Sb_1$. See Appendix 1A for a description of the mutations in this stock. In the crossing procedures the second chromosome containing Cy and the third chromosome containing Me were used as balancers as they are known to prevent crossover approximately 95% of the time. Starting with single pairs, the scheme followed for obtaining homozygous

chromosome stocks is illustrated in Figure 1. A heterozygote obtained from the first single pair mating with the marker stock was backcrossed to the marker stock in order to obtain several progeny containing the same second or third chromosomes. Appropriate brother-sister matings were then performed to obtain the desired homozygous chromosomes. When it was desired to combine homozygous chromosomes originally obtained from different flies, the procedure shown in Figure 2 was employed. Stocks containing the required homozygous chromosomes with dominant markers on the other chromosomes were crossed. F_2 progeny were then obtained from crosses between heterozygotes bearing the Cy and Me markers. From among the F_2 , flies were selected which contained neither dominant marker and hence were homozygous for both second and third chromosomes.

Screening Procedures

In order to test for crc phenocopy susceptibility, larvae between the ages of 65 and 70 hours must be fed a glucosamine solution (Rizki, 1960). Timed larvae were collected in the following manner. Adult flies between the ages of 4 to 10 days were released in a clean 1/2 pint bottle and females were allowed to lay eggs on a fiber coffee spoon containing a mound cream of wheat media smeared with a paste of yeast, water, and honey. Twenty-two to twenty-four hours later the spoons were removed from the bottle, the hatched larvae were

Key for Figs. 1 and 2

Dominant marker second chromosome	
Dominant marker second chromosome	
Dominant marker third chromosome	
Dominant marker third chromosome	
Second chromosome to be isolated in homozygous condition	
Third chromosome to be isolated in homozygous condition	

FIG. 1

Isolation of homozygous second and third chromosomes

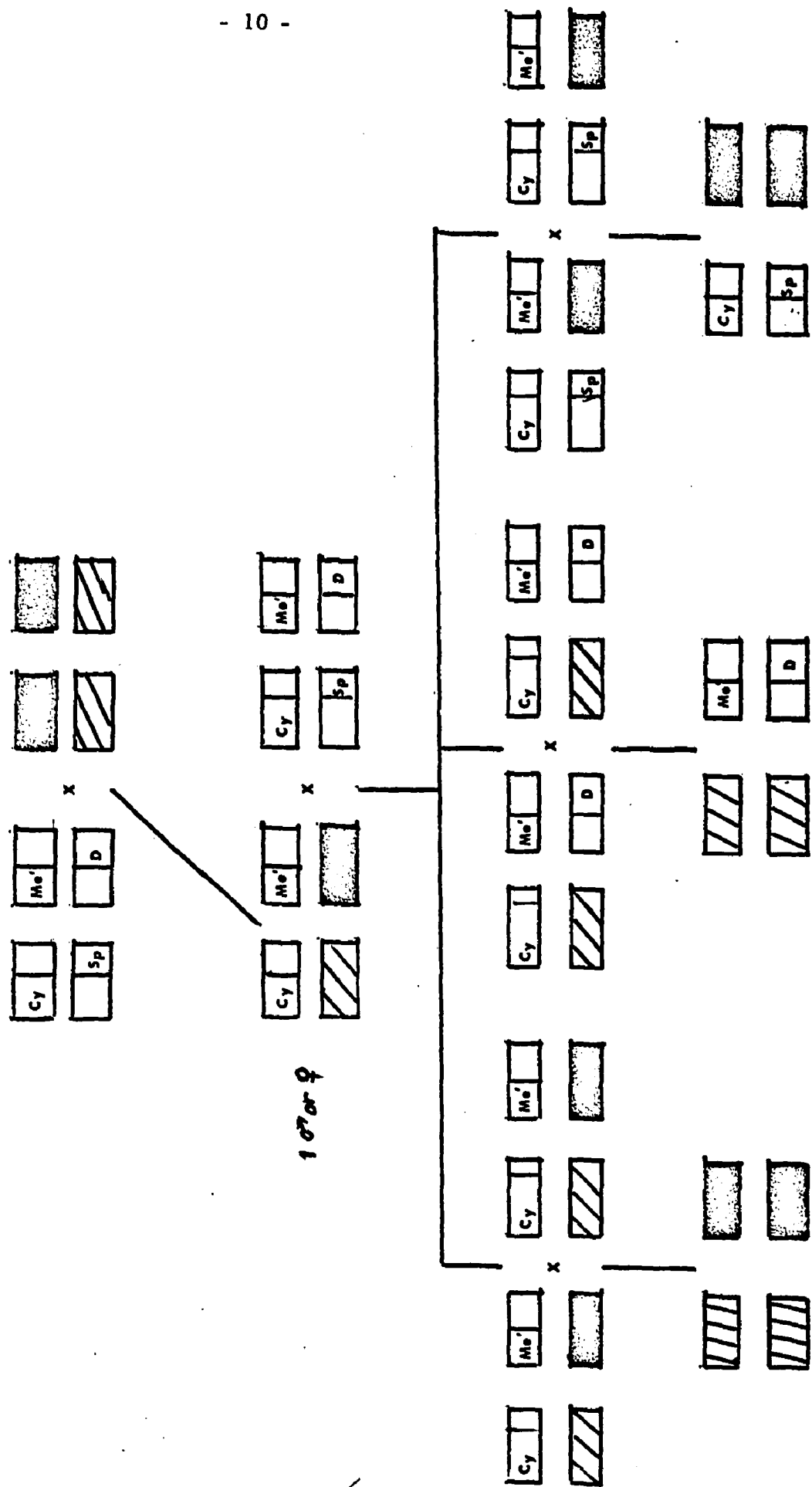
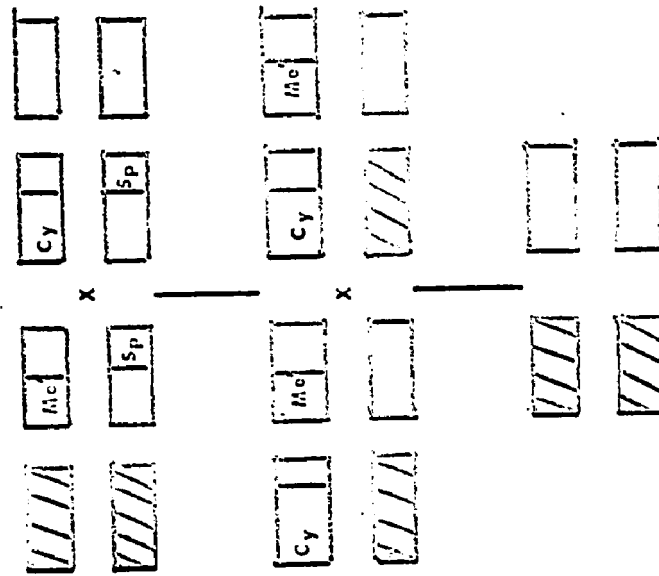


FIG. 2
Combining homozygous second and third chromosomes from
different lines



cleared from the spoon with a dissecting needle. Approximately two hours later the larvae which had hatched during the interval were collected and transferred to a crystal dish containing a 3/8 inch layer of cream of wheat media, seeded with a yeast solution and covered with a filter paper lined petri dish top. The larvae were timed from the midpoint of the 2-hour interval, ± 1 hour, and allowed to develop in a Precision Scientific Company incubator held at $25^{\circ}\text{C} \pm 1^{\circ}$. The maximum number of larvae per dish was about 90, the usual number was 80.

Third instar larvae were collected between 65 and 67 hours by flotation with concentrated NaCl solution. Larvae which had burrowed into the substrate were loosened with a dissecting needle. The larvae were poured into a 140 ml beaker from which the NaCl solution was removed with a pipette. A 5% Clorox solution was next poured into the beaker in order to remove adhering microbes which might otherwise metabolize glucosamine, thus altering its concentration. The larvae were rinsed 5 times in 140 ml of distilled H_2O to remove any detectable odor of Clorox. Next, the larvae were placed in 60 x 15 mm glass petri dishes in which 1/4 teaspoon of Whatman's cellulose powder CF2 was placed along with 1.5 ml. treatment solution. In order to reduce fungal infection, the petri dishes and pipettes were heat sterilized at 168°C for 1/2 hour. The camel hair brushes used to transfer larvae from the beaker to the petri dishes were immersed in 70% ethyl alcohol for several minutes and then thoroughly rinsed in a stream of

distilled H₂O.

About 25 larvae were treated either with distilled H₂O (a control) or 5% glucosamine HCl. This concentration was found (Rizki, 1960) to give the optimal amount of phenocopy without causing appreciable mortality. After the petri dish lid was lined with 1/2 a Kleenex tissue which prevented larvae from escaping, the dish was placed in a 25°C±1° incubator exclusively used for this purpose. A tray of water was routinely kept in the bottom of the incubator to maintain a high humidity and to avoid dessication.

The larvae pupate in the petri dish at approximately 96 hours of age. Six to seven days after treatment, when adult flies began to emerge from their pupa cases, the animals were scored. Each pupa was classified into one of three categories: normal, affected, or crc phenocopy. The normal classification included all pupae whose larval mouth parts were pushed to the side by the cephalic complex during head eversion. The affected group included those pupae in which larval mouthparts remained imbedded in larval tissue and where no developing wings or leg primordia were apparent. The typical "affected" animal may be considered in a developmental arrest prior to the time of head eversion. The crc phenocopy was recognized by the pigmented eyes within the thorax. When pigmentation did not occur, additional criteria were used. The uneverted cephalic complex combined with typical crc

wings or legs was considered indicative of a phenocopy response. In all categories sex was determined by inspection of the genital region or the sex comb region, where possible.

In subsequent analysis only the crc phenocopy category was used as an indicator of phenocopy susceptibility. Appendix 8A contains raw data concerning the numbers of organisms in the normal, affected and phenocopy classifications in our experiments.

RESULTS

Table 1 presents the pooled data of many experiments in which glucosamine induced phenocopy was measured in seven laboratory strains. The individual experiments were carried out at different times and within each strain there was considerable day to day variation in response. Nevertheless, these data indicate the average differences among the seven strains and provided the initial guide line for selection of the four most promising strains for detailed investigation. The strains carrying the alleles w^h_{sn}³ (white honey, singe³), w^{bl} (white blood), e¹¹ (ebony¹¹) and the wild type strain Ore-R were chosen for further study. Separate experiments were then undertaken to determine the relative ranking of these strains with respect to glucosamine response; data and statistical analysis are presented in Table 2. It is based upon observations made on several days of experimentation since different genotypes were not necessarily screened on the same day due to physical limitations. A one way analysis of variance with unequal observations per treatment indicated significant differences among strain means at the five percent level. As the frequency distribution of the data was approximately normal, valid significance tests did not suggest transformation of the data. Duncan's Multiple Range Test was applied to determine which differences among means were significant at this level. Those strains which are underlined by a common bar were not statistically different in their average response.

Table 1

Phenocopy Susceptibility in Seven
Strains of Drosophila melanogaster

Strain	Number of Pupae (N)	% <u>crc</u> phenocopy (P)	s*
w ^h sn ³	335	8.75	1.5
w ^{bl}	394	9.17	1.4
w	465	4.17	0.9
v ^k	349	2.37	0.8
v ^{36f}	290	0.0	0.0
Ore-R	173	4.74	1.6
e ^{ll}	324	23.93	2.4

$$s^* = \sqrt{\frac{pq}{N}}, \text{ where } q = 100 - p$$

Table 2

Comparison of Phenocopy Susceptibility Among Four Strains of *Drosophila melanogaster*

Run*	Strain			
	Ore-R	e ¹¹	w ^{bl}	w ^h sn ³
	percent phenocopy response			
1	19.5	25.5	49.0	26.2
2	1.9	31.3	38.9	27.2
3	2.7	51.3		15.5
4	19.8	—	—	—
Average	10.4	36.0	44.0	23.0

*Experiments within a run were not necessarily performed on the same day.

Source	df	Sums of Squares	Mean Squares	Expected Mean Squares	F	p
Strains	3	1880.29	626.76	$\sigma^2_{\epsilon} + K\sigma_s^2$	6.2	.025 > p > .01
Within Strains	8	809.08	101.14	σ^2_{ϵ}		

$$K = \frac{\sum_{i=1}^s n_i - \sum_{i=1}^s (n_i)^2 / N}{s} = 2.94$$

s = number of strains

n_i = number of runs in strain i

N = total number of runs in all strains

Duncan's Multiple Range Test:

w^{bl} e¹¹ w^hsn³ Ore-R

Upon establishing variation in phenocopy response among the four strains, an investigation was made of the genetic basis of this response. The possibility of sex dependence could not be explored from an examination of the sex ratios of phenocopied individuals since these individuals often died prior to the pigmentation of external sex structures and sex could not be determined. Therefore, reciprocal crosses between pairs of strains were made so that any difference in response between reciprocal crosses could be attributed to the x-chromosome or to maternal effects. The results of these studies are presented in Table 3. None of the reciprocals gave significantly different results, thus we fail to detect an x-chromosome or a maternal effect contribution to the observed frequency difference of phenocopy susceptibility. On the basis of the above results it would appear that the laboratory strains under study differed significantly in phenocopy response and these differences did not appear to be strongly influenced by sex linked genes.

It should be noted that no definitive comparisons between the quantitative response of the F_1 progeny in Table 3 and parental strains in Table 2 should be made as these data were gathered under quite different laboratory conditions. However, a comparison of the relative magnitudes of these responses might suggest whether or not one might anticipate that genes at loci determining the reaction of the responsive system to glucosamine are allelic. That is, allelism might be expected

Table 3

Phenocopy Analysis of Reciprocal Crosses

♀	♂	% <u>crc</u> phenocopy	χ^2 *	Average F ₁ response
w ^h sn ³	x e ¹¹	15.62	0.00 ^{N. S.}	15.60
e ¹¹	x w ^h sn ³	15.57		
e ¹¹	x w ^{bl}	27.24	1.18 ^{N. S.}	23.51
w ^{bl}	x e ¹¹	19.78		
e ¹¹	x Ore-R	11.87	0.24 ^{N. S.}	10.75
Ore-R	x e ¹¹	9.62		
w ^h sn ³	x w ^{bl}	20.23	0.73 ^{N. S.}	18.65
w ^{bl}	x w ^h sn ³	17.06		

N. S. = not significant at the 0.10 level

*Tests difference between % phenocopy of reciprocal crosses.

in cases where the F_1 phenocopy response is equal to one parental response or where the F_1 response lies between the two parental responses. The averages presented in Table 2 and Table 3 are relevant to this point. It may be seen that with the exception of Ore-R, all parental strains showed a phenocopy responsiveness equal to or greater than the F_1 progeny. This suggests that at least in some cases different loci are involved in determining the phenocopy response in different strains. Furthermore, the low F_1 response relative to parentals suggests the presence of dominant gene action. On the other hand, in a comparison of ranks generated by averaging the parental strain responses and the corresponding F_1 responses the concordance among these ranked series as indicated in Table 4 is interesting and may mean that some similarities of an additive nature must also exist among these strains.

As there was considerable variability in phenocopy responsiveness obtained within a given strain which was screened on different days (see Table 2) we wished to know whether the variability was due to environmental factors or to genetic heterogeneity. Experiments were designed not only to evaluate the genotypic homogeneity but also the contributions of the second and third chromosomes to phenocopy response. However, statistical tests utilized in analysis of these experiments assume that the variable is continuous and approximately normally

Table 4

Rank of Average Responses of Two Parental Strains and the
Average Value Obtained for Corresponding F_1 Hybrids

Parentals	Percent $\frac{P_1 + P_2}{2}$	Rank	$P_1 \times P_2 = F_1$	Rank
e ¹¹ Xw ^{bl}	40.0	1	23.5	1
w ^{bl} Xw ^h _{sn3}	33.5	2	18.7	2
e ¹¹ Xw ^h _{sn3}	29.5	3	15.6	3
Ore-RXe ¹¹	16.7	4	10.8	4

distributed. In most of the experiments the observations (percent phenocopy) were not normally distributed. That is, they did not approximate a bell shaped curve. A transformation to a scale which would approximate a normal distribution was necessary to perform valid F tests of significance. Since the original data showed a clustering near zero the arcsin of the square root of the percentage phenocopy was considered and found to give the appropriate transformation (Snedecor, 1967, p. 327).

To evaluate the homogeneity within the strains and the contribution of the second and third chromosomes to the responsive system the latin square design was utilized since it permits the experimenter to evaluate the experiment carried out on different days without biasing the evaluation of chromosomal effects and their interactions.

In general a replicated 3 x 3 latin square experimental design where the second chromosome, third chromosome and runs correspond to rows, columns, and letters respectively (see Peng, 1967) was used to estimate effects described by the mathematical model below.

$$Y_{ijkm} = \mu + \alpha_i + \beta_j + Ru_k + \alpha\beta_{ij} + R_m + c(x_{ijkm} - \bar{x}) + \epsilon_{ijkm}$$

Y_{ijkm} is the transformed value of the proportion of phenocopy observed when glucosamine HCl was fed to a given genotype representing the i th second and j th third chromosomes in the k th run and the m th replica-

tion; μ is the overall mean of the $Y_{ijk\mu}$; α_i term refers to the contribution to phenocopy of the i th second chromosome; $i = 1, 2, 3$, while the β_j term refers to the contribution to phenocopy of the j th third chromosome $j = 1, 2, 3$. The Ru_k are the effects of the runs on phenocopy where $k = 1, 2, 3$. As it was impractical to screen all nine genotypes on one day, the screening was conducted on three different days (designated runs) using three different genotypes for each run in the usual latin square layout (see Fig. 3, p. 28).

The $\alpha\beta_{ij}$ term is taken as the contribution to phenocopy of the interaction (between the i th second chromosome and the j th third chromosome) adjusted for run differences. Although chromosome effects are orthogonal with run effects in this design, the same cannot be said of the measurement of their interaction effect. In this case, however, variation due to interaction effects may be partitioned into variation due to runs and variation due to interaction adjusted for runs. Computationally, the adjusted interaction sums of squares was obtained as the difference between the interaction sums of squares ignoring runs and the runs sum of squares.

To reduce the experimental error, replicates (R_m , $m = 1, 2$) and deviations attributable to water (control differences) were also considered. c is the linear regression coefficient for the dependence of

Y_{ijkm} on the effect of water. Each covariate, x_{ijkm} is the transformed value of the phenocopy observed on the water control for the i th and j th chromosome, k th run and the m th replication. $\bar{x}$ is the mean of all x_{ijkm} . The ϵ_{ijkm} measures the random deviation of Y_{ijkm} not attributable to other sources of variation in the model.

Replication, run, and error effects were taken to be a random sample of all possible such effects whereas chromosome effects were considered random when an estimate of variability in the material sampled was desired and fixed when only differences among the specific chromosomes represented in the study were considered. The experiments were planned such that every replicate of every genotype was to contain approximately 100 animals (in four dishes containing 25 larvae per dish) treated with glucosamine HCl and 100 treated with water. Owing to different egg laying and hatching frequencies for the different genotypes, the desired numbers of animals were not always available. In some cases a zero phenocopy percentage was based on less than fifty observations; in others statistical problems involving the estimation of a missing value and possible binomial variation due to proportions based on unequal numbers were also encountered. Therefore where zero phenocopy percentages were based on less than 50 pupae, transformed values were adjusted according to Bartlett (1947) by a factor of $1/4n$ where n is the number of animals scored for the ij th

genotype in the mth replicate. The missing value MV was estimated according to Snedecor (1967):

$$MV = \frac{a(R+C+T) - 2G}{(a-1)(a-2)}$$

where

a = number of rows or columns
R = marginal total of the row with the missing value
C = marginal total of the columns with the missing value
T = run total of the run in which the missing value occurred
G = grand total

To determine if the data should be weighted due to basing proportions on unequal numbers, the proportion of the total variance due to binomial variance was computed according to Cochran (1943). If this value is greater than 50%, weighting is advisable. The suggested computations were performed for the experiment with the greatest differences between numbers used in different cells, $e^{11}-w^{b1}$. As less than 1% of the variance was found to be binomial, the idea of weighting the data was discarded. (See Appendix 7A for these calculations.)

The latin-square design described above was used in two ways to answer three different questions. The first question was that of the degree of genetic variability of response within an inbred strain. The second question was that of whether the second and third chromosomes contributed equally to phenocopy susceptibility. As the preliminary experiments indicated that the x chromosome did not affect phenocopy

susceptibility detectably, its role was not explored further. The final question was that of the nature of the gene action responsible for phenocopy susceptibility. These inquiries were answered by utilization of the 3 x 3 latin-square design under different assumptions. One is based on the random effects model to answer question one and the mixed effects model assumptions were used to answer the second and third questions.

Random effects model (REM): In this model the i th variable is assumed drawn from an infinite population with a zero mean and a variance σ_i^2 . The model is appropriate where information concerning population variance is desired. In our experiments the random effects model was used to test the null hypothesis that there is no difference in phenocopy susceptibility among second (or third) chromosomes of a given strain.

Mixed effects model (MEM): In this case the chromosomal effects in the model are fixed effects which may be estimated while the replication, run, and error effects are taken to be random. In the present experiments the mixed effects model was used to estimate the contribution to phenocopy response of particular second and third chromosomes, the null hypothesis being that the chromosomes in this sample do not contribute to phenocopy response.

To test for genetic homogeneity within a given strain homozygous second and third chromosomes from three females were derived

as previously described and combined in all ways. This procedure was carried out for e¹¹ a highly responsive strain and for Ore-R a low responding strain. The design is illustrated in Figure 3. Note that all chromosomes are homozygous and that each second chromosome is combined with each of three third chromosomes and vice versa. In testing for genetic homogeneity within strains the chromosomal effects are considered to be random variables with a mean of zero and a variance σ^2 . The expected mean squares (EMS) generated using this assumption are shown in Table 5. The mean square (MS) for interaction comprises the denominator of the F ratio which is used to test for the significance of chromosomal variation under the random model assumptions.

The same experimental data can be used to estimate the contributions of second and third chromosomes under the mixed effects model assumptions. The EMS under these assumptions are given in Table 5 also. It will be noted that here the denominator of the F ratio consists of the mean squares (MS) for error. These experiments were analyzed utilizing the standard analysis of covariance procedure (Steel and Torrie, 1960). Such an analysis adjusts experimental error and treatment means for the linear effect of water on phenocopy response prior to tests of significance.

Fig. 3

Experimental Design to Test For the Homogeneity
of Phenocopy Response in a Strain

		3rd chromosome		
		a/a	b/b	c/c
2nd chromosome	a/a	a/a,a/a Run 1	a/a,b/b Run 2	a/a,c/c Run 3
	b/b	b/b,a/a Run 3	b/b,b/b Run 1	b/b,c/c Run 2
	c/c	c/c,a/a Run 2	c/c,b/b Run 3	c/c,c/c Run 1

Key:

a Chromosome derived from
strain a

b Chromosome derived from
strain b

c Chromosome derived from
strain c

Table 5

Analysis of Covariance for Phenocopy Susceptibility for e^{11}

Source	df	EMS Random Effects Model	EMS Mixed Effects Model	Y adjusted for X		Random Effects Model	Mixed Effects Model
				SS	MS	F	F
error	7	σ^2_ϵ	σ^2_ϵ	230.15	32.88		
2nd chromosome ad- justed for H ₂ O	2	$\sigma^2_\epsilon + 2\sigma^2_{\alpha\beta} + 6\sigma^2_\alpha$	$\sigma^2_\epsilon + 3\sum_{i=1}^3 \alpha_i^2$	294.84	147.42	2.39 N.S.	4.48*
3rd chromosome ad- justed for H ₂ O	2	$\sigma^2_\epsilon + 2\sigma^2_{\alpha\beta} + 6\sigma^2_\beta$	$\sigma^2_\epsilon + 3\sum_{j=1}^3 \beta_j^2$	298.90	149.45	2.42 N.S.	4.55*
2nd x 3rd chromo- some adjusted for H ₂ O and runs	2	$\sigma^2_\epsilon + 2\sigma^2_{\alpha\beta}$	$\sigma^2_\epsilon + \sum_{i=1}^3 \sum_{j=1}^3 \alpha\beta_{ij}^2$	123.59	61.80	1.88 N.S.	1.88 N.S.
run adjusted for H ₂ O	2	$\sigma^2_\epsilon + 2\sigma^2_{\alpha\beta} + 6\sigma^2_{Ru_k}$	$\sigma^2_\epsilon + 6\sigma^2_{Ru_k}$	607.43	303.72	4.90*	9.24**
replication adjusted for H ₂ O	1	$\sigma^2_\epsilon + 9\sigma^2_{R_m}$	$\sigma^2_\epsilon + 9\sigma^2_{R_m}$	40.07	40.07	1.22 N.S.	1.22 N.S.

* = P(larger F) < 0.10

** = P(larger F) < 0.05

NS = Not Significant

The experimental results are given in Tables 5 and 6. Table 5 shows that for e¹¹ there is no significant variation among second or third chromosomes of this strain (under the random model), that is, the probability of observing F ratios of 2.39 for chromosome two, 2.42 for chromosome three or 1.88 for chromosomal interaction by chance is greater than one in ten for each. However when the mixed effects model (chromosome averages free of interaction effects) is used on the same data to determine if the second or third chromosomes of this sample contribute to phenocopy susceptibility we find that both second and third chromosomes are contributing to some extent. Each of the observed F ratios of 4.48 and 4.55 for second and third chromosomes respectively would occur by chance less than one time in ten. Furthermore as the second and third chromosomes showed similar deviations from the overall mean (e. g., mean squares of 147.42 and 149.45 respectively) it appears that they contribute about equally to variability in phenocopy response. There was no significant interaction between second and third chromosomes.

In the experiment involving chromosomes derived from the Ore-R stock the interaction term was so small it was felt that the mean square for error was a better estimate of error for testing the significance of main chromosomal effects (Dixon and Massey, 1957, p. 177). For this reason, only the mixed model was utilized and as

Table 6

Analysis of Covariance for Phenocopy Susceptibility of Ore-R					
Source	df	EMS Mixed Effects Model	Y adjusted for X		Mixed Effects Model F
			SS	MS	
error	7	σ^2_{ϵ}	120.51	17.22	
2nd chromosome adjusted for H ₂ O	2	$\sigma^2_{\epsilon} + 3 \sum_{i=1}^3 \alpha_i^2$	41.69	20.85	< 1.21 N.S.
3rd chromosome adjusted for H ₂ O	2	$\sigma^2_{\epsilon} + 3 \sum_{j=1}^3 \beta_j^2$	19.13	9.57	< 1 N.S.
2nd x 3rd chromosome ad- justed for H ₂ O and runs	2	$\sigma^2_{\epsilon} + \sum_{i=1}^3 \sum_{j=1}^3 \alpha\beta_{ij}^2$	16.03	8.02	< 1 N.S.
run adjusted for H ₂ O	2	$\sigma^2_{\epsilon} + 6\sigma^2 R_{uk}$	22.83	11.42	< 1 N.S.
replication adjusted for H ₂ O	1	$\sigma^2_{\epsilon} + 9\sigma^2 R_m$	15.67	15.67	< 1 N.S.

N. S. = Not significant

can be seen from Table 6 no phenocopy response was detectable (observed F values of less than one occur with a probability greater than 0.25). Although no detectable variation in phenocopy response of chromosome was found in the e¹¹ and Ore-R strains the possibility exists that these results were due to sampling error as only six chromosomes from each strain were sampled.

The above 3 x 3 Latin-square design was used to answer the third question concerning the qualitative nature of the gene action responsible for phenocopy susceptibility. It will be recalled that preliminary experiments indicated the noninvolvement of the x chromosome as well as the presence of dominant and additivity among loci. Therefore the experiment shown in Figure 4 was designed to evaluate the gene action associated with second and third chromosomes. Homozygous second and third chromosomes from each of two inbred strains (designated a and c and their heterozygotes H) are combined in all possible ways. Because the response of particular chromosomes was of interest the mixed effects model (with fixed chromosomal effects and random environmental effects) was appropriate. An important aspect of this design is that a significant chromosomal effect can be partitioned into linear (additive) and quadratic (dominant) components which can be tested for statistical significance. The linear components are the differences between the homozygous chromosomes and

Fig. 4

Experimental Design for Elucidating the Genetic
Basis of the Phenocopy Response

		3rd Chromosome		
		a/a	a/c	c/c
2nd chromosome	a/a	a/a,a/a Run 1	a/a,H Run 2	a/a,c/c Run 3
	a/c	H,a/a Run 3	H,H Run 1	H,c/c Run 2
	c/c	c/c,a/a Run 2	c/c,H Run 3	c/c,c/c Run 1

Key:

a = Chromosome derived from
strain a

c = Chromosome derived from
strain c

H = Heterozygote a/c

the quadratic effects measure the degree to which the heterozygote differs from $1/2$ the sum of the homozygous parentals. The EMS in this type of experiment are the same as those in Table 6. Again the standard analysis of covariance procedure was utilized to analyze the results. Partitioning of the mean squares into single degree of freedom contrasts was carried out where appropriate according to the method of Cochran and Cox (1967, p. 89). In this method the sums of squares for error are correct, however, the sums of squares for contrasts are inflated thus preventing their sum equalling that of the treatment sums of squares. Three experiments utilizing this design were performed, in one e¹¹ and w^hsn³ chromosomes were used, in another, e¹¹ and Ore-R chromosomes were used, the third experiment involved e¹¹ and w^{b1} chromosomes. It was hoped that these experiments would provide a sufficiently broad sample of the possible types of gene action operating in the phenocopy response system to assess the nature of the genetic mechanisms involved.

Turning to the results of these experiments, Table 7 shows the results of all possible combinations of an e¹¹ second and third chromosome with an Ore-R second and third chromosome. In this case there is a significant phenocopy response from second and third chromosomes as well as a significant interaction between the second and third chromosomes. (The observed F values of 13.68, 18.18 and 5.37 for

Table 7

Analysis of Covariance for Phenocopy Susceptibility of Combinations of Chromosomes Derived from ell and Ore-R

Source	df	EMS Mixed Effects Model	Y adjusted for X		Mixed Effects Model F
			SS	MS	
error	7	$\sigma^2 \epsilon$	158.88	22.70	
2nd chromosome adjusted for H ₂ O	2	$\sigma^2_{\epsilon+3 \sum_{i=1}^3 \alpha_i^2}$	620.99	310.50	13.68***
3rd chromosome adjusted for H ₂ O	2	$\sigma^2_{\epsilon+3 \sum_{j=1}^3 \beta_j^2}$	825.34	412.67	18.18***
2nd x 3rd chromosome adjusted for H ₂ O and runs	2	$\sigma^2_{\epsilon+ \sum_{i=1}^3 \sum_{j=1}^3 \alpha \beta_{ij}}$	243.66	121.83	5.37***
run adjusted for H ₂ O	2	$\sigma^2_{\epsilon+6 \sigma^2 Ru_k}$	232.89	116.45	5.13***
replication adjusted for H ₂ O	1	$\sigma^2_{\epsilon+9 \sigma^2 R_m}$	27.43	27.43	1.21 N.S.

*** = P(larger F) < 0.01

N. S. = Not Significant

second chromosomes, third chromosomes and second x third chromosomal interactions respectively have a $P < 0.01$.) Unfortunately, run effects (e. g. , error introduced due to different days of experimentation) were also significant in this experiment. Since run effects and interaction effects are confounded in this design, the type of epistatic effects contributing to interaction could not be evaluated by single degree of freedom contrasts without introducing bias from the run component. To overcome this difficulty a table of phenocopy responses adjusted for water and runs was prepared in the hope that a comparison of the mean responses might suggest the nature of the epistatic effects. An examination of Table 8 will reveal that parental homozygotes (corner cells 9.49, 36.31, 22.26 and 35.00) have a uniformly higher phenocopy susceptibility than their heterozygous progeny (8.33, 2.98, 2.66, 16.58 and 7.87) suggesting the presence of dominance. The phenocopy response of the double heterozygote (2.66) was similar to that of the lowest value obtained for a single heterozygote (2.98).

When the same type of experiment was performed with e¹¹ and w^hsn³ chromosomes, Table 9, again both second and third chromosomes and their interaction (with observed F ratios of 7.10, 5.13 and 4.16 respectively) were significant (with a $P < 0.05$). In this case, because the run component was not significant, the interaction could be pooled with runs and investigated further. Among the possible types

Table 8

Means of Transformed and Adjusted Observations

		3rd chromosome		
2nd chromosome		Ore-R	$e^{11}/\text{Ore-R}$	e^{11}
	Ore-R	9.49	8.33	36.31
	$e^{11}/\text{Ore-R}$	2.98	2.66	16.58
	e^{11}	22.26	7.87	35.00

$$\bar{Y}_{ij} \text{ adjusted} = \bar{Y}_{ij} - c(\bar{x}_{ij} - \bar{\bar{x}}) - (Ru_k - \bar{Ru})$$

c = regression of Y on x

Table 9

Analysis of Covariance for Phenocopy Susceptibility of Combinations of Chromosomes Derived from e_{11} and w_{sn}^3

Source	df	EMS Mixed Effects Model	Y adjusted for X		Mixed Effects Model F
			SS	MS	
error	7	$\sigma^2 \epsilon$	87.53	12.50	
2nd chromosome adjusted for H_2O	2	$\sigma^2 \epsilon + 3 \sum_{i=1}^3 \alpha_i^2$	177.37	88.69	7.10**
3rd chromosome adjusted for H_2O	2	$\sigma^2 \epsilon + 3 \sum_{j=1}^3 \beta_j^2$	128.34	64.17	5.13**
2nd x 3rd chromosome adjusted for H_2O	4	$\sigma^2 \epsilon + \sum_{i=1}^3 \sum_{j=1}^3 \alpha\beta_{ij}^2$	209.67	51.97	4.16**
average linear 2x linear 3 response adjusted for H_2O	1		13.17	13.17	1.10 N.S.
average quadratic 2x quadratic 3 response adjusted for H_2O	1		121.52	121.52	9.71**
average quadratic 2x linear 3 response adjusted for H_2O	1		9.75	9.75	< 1 N.S.
average linear 2x quadratic 3 response adjusted for H_2O	1		3.94	3.94	< 1 N.S.
replication adjusted for H_2O	1	$\sigma^2 \epsilon + 9\sigma^2 R_m$	5.71	5.71	< 1 N.S.

** = $P(\text{larger } F) < 0.05$

N.S. = Not significant

of interaction, only dominant x dominant effects were found significant. If the adjusted phenocopy responses (Table 10) are examined in this experiment one again notices that the heterozygotes have lower phenocopy response than the parental homozygotes. It is interesting that the double heterozygote (center cell) has a higher response than the single heterozygotes.

In the case of the e¹¹ and w^{bl} experiment (Table 11) second and third chromosomes appear to be contributing factors to phenocopy response. F values of 3.72 for second chromosomes and 4.48 for third chromosomes each have a $P < 0.1$. Interaction was not significant with an F ratio of less than 1. Since no interaction could be detected, the main chromosomal effects may be broken down into additive and dominant components directly from marginal totals. It may be seen that only dominant effects were found significant as the F ratios of 5.18 and 6.15 for dominant components would occur by chance less than five times in a hundred. The distribution of the dominant genes on both chromosomes may be ascertained in part by examining the adjusted phenocopy responses in the experiment as shown in Table 12. Notice that when homozygotes in the corner cells of the table (20.38, 10.82, 17.96 and 17.39) are compared with their heterozygotes (9.22, 10.38, 7.50 and 9.11) the heterozygotes are uniformly lower in phenocopy susceptibility than either parental genotype. Of further interest is the fact

Table 10

Means of Transformed and Adjusted Observations

		3rd chromosome		
2nd chromosome		e ¹¹	e ¹¹ /w ^h _{sn} ³	w ^h _{sn} ³
	e ¹¹	20.69	12.62	25.05
	e ¹¹ /w ^h _{sn} ³	8.83	13.95	13.89
	w ^h _{sn} ³	15.96	7.59	15.18

$$\bar{Y}_{ij} \text{ adjusted} = \bar{Y}_{ij} - c(\bar{x}_{ij} - \bar{\bar{x}})$$

c = regression of Y on x

Table 11

Analysis of Covariance for Phenocopy Susceptibility of Combinations of Chromosomes Derived from ell and wbl

Source	df	EMS Mixed Effects Model	Y adjusted for X		Mixed Effects Model F
			SS	MS	
error	7	σ^2_ϵ	139.82	19.97	
2nd chromosome adjusted for H ₂ O	2	$\sigma^2_\epsilon + 3 \sum_{i=1}^3 \alpha_i^2$	148.65	74.33	3.72*
linear response adjusted for H ₂ O	1		32.30	32.30	1.32 N.S.
quadratic response adjusted for H ₂ O	1		119.51	119.51	5.18**
3rd chromosome adjusted for H ₂ O	2	$\sigma^2_\epsilon + 3 \sum_{j=1}^3 \beta_j^2$	178.90	89.45	4.48*
linear response adjusted for H ₂ O	1		47.93	47.93	2.40 N.S.
quadratic response adjusted for H ₂ O	1		150.31	150.31	6.15**
2nd x 3rd chromosome adjusted for H ₂ O and runs	2	$\sigma^2_\epsilon + 3 \sum_{i=1}^3 \sum_{j=1}^3 \alpha\beta_{ij}^2$	31.61	15.81	< 1 N.S.
run adjusted for H ₂ O	2	$\sigma^2_\epsilon + 6 \sigma^2_{R_{uk}}$	389.32	194.66	9.75**
replication adjusted for H ₂ O	1	$\sigma^2_\epsilon + 9 \sigma^2_{R_m}$	0.01	0.01	< 1 N.S.

* = P(larger F) < 0.10

** = P(larger F) < 0.05

N.S. = Not significant

Table 12

Means of Transformed and Adjusted Observations

3rd chromosome

2nd chromosome	3rd chromosome			
	e ^{ll}	e ^{ll} /w ^{bl}	w ^{bl}	
	e ^{ll}	20.38	9.22	10.82
	e ^{ll} /w ^{bl}	10.38	8.22	7.50
	w ^{bl}	17.96	9.11	17.39

$$\bar{Y}_{ij} \text{ adjusted} = \bar{Y}_{ij} - c(\bar{x}_{ij} - \bar{\bar{x}}) - (Ru_k - \bar{Ru})$$

c = regression of Y on x

that the double heterozygote is of the same order of magnitude as the single heterozygotes.

DISCUSSION

A sine qua non of genetic experimentation has always been that under a given set of environmental conditions genes are responsible for and in some manner control the phenotypic variation of the organism. Several other features of the genetic control of phenotype have also become apparent. It was found that wild populations contained tremendous variability in terms of the genes used to control a particular phenotype. Furthermore, it was noted that heterozygosity on a genetic level usually resulted in phenotypic stability and that homozygosity often led to phenotypic variation (Lerner, 1954). Such findings suggested that genes not only regulate the production of the phenotype but also buffer developmental pathways protecting them from upset due to environmental changes. Such an interpretation seems even more valid when considering pheno-deviation caused by specific environmental agents which can be shown to cause abnormalities with different frequencies in different inbred strains (Landauer, 1957; Goldschmidt and Piternick, 1957 a, b).

As previously stated, this study was undertaken in the hope that an analysis of the distribution and behavior of genes controlling the buffering of a particular developmental pathway might provide clues

to the general manner in which developmental homeostasis is genetically controlled.

Genetic Variability of Phenocopy
Response Among Strains

In order to explore the control of glucosamine phenocopy response in Drosophila melanogaster four stocks of varying phenocopy susceptibility were studied. An analysis of these stock showed that the differences in phenocopy potential between stocks is greater than the variability in phenocopy response within stocks indicating a genetic component of variation. Furthermore, the stocks could be ranked on the basis of decreasing phenocopy susceptibility as follows:

w^{bl} > e^{ll} > w^h_{sn}³ > Ore-R. From this rather crude comparison between the magnitude of susceptibility of parent stocks with their F₁ hybrid offspring the following may be inferred. Since in general, the F₁ response was lower than either parent one suspects that a number of recessive mutations are involved but that these mutations are distributed differently among the stocks and consequently their effects are masked in the F₁. As all hybrids showed a phenocopy susceptibility the same or higher than the Ore-R stock we may infer that at least among the stock tested, Ore-R represents a lower limit of susceptibility. Thus, Ore-R may be considered to contain relatively few "responsive" mutant alleles.

Genetic Variability of Phenocopy
Response Within Strains

The next question considered was that of whether a given laboratory stock would be genetically homogeneous with respect to genes controlling the phenocopy response. That is, does every member of the stock have an equal probability of being phenocopied? This question was examined by determining the phenocopy response of randomly selected viable second and third chromosomes combined in all possible ways. These studies were carried out in two strains, e¹¹ and Ore-R. Since there was no significant difference among second chromosomes or among third chromosomes and no interaction between these chromosomes could be found in the e¹¹ stock, we may conclude that the variance of the frequency of phenocopy susceptibility among zygotes is so low that significant variability in the population is not revealed in a six chromosome sample. Consequently it appears that for this stock the genetic basis of crc phenocopy responsive system is more or less the same in different organisms and that the 30% response characteristic of the e¹¹ strain represents the genetically determined susceptibility risk for all individuals of this stock. The phenomenon of phenocopy susceptibility may then be a manifestation of the penetrance of phenocopy susceptible genes. The term penetrance was introduced by Timofeef-Resovsky (1926) to describe the often encountered situation in which a particular mutant gene is expressed less than 100% of the

time in stocks homozygous for the gene. As a given member of a population may or may not show the mutant phenotype there appears to be a developmental-physiological threshold which determines which phenotype will be manifested by the organism (Sang, 1963). The crc phenocopy susceptible system would seem to be "explained" in the same way that penetrance phenomena have been. In the crc case, however, apparently several mutant alleles rather than a single allele are involved.

As explained previously, we were unable to utilize the REM to investigate genetic homogeneity in the Ore-R experiment. When the MEM was used to test for significant chromosomal contributions, however, none were found. From this information we can only assume that at such low levels of phenocopy susceptibility variations in phenocopy susceptibility among chromosomes are difficult to detect.

Specific Chromosomal Involvement

We now turn to the question of the distribution of phenocopy susceptible genes on the second and third chromosomes. As noted above, the mean squares for both second and third chromosomes in the e¹¹ stock were about equal suggesting that these chromosomes contribute equally to phenocopy susceptibility. It is interesting to note that there are 108 crossover units in the second chromosome linkage map and 110 crossover units in the third chromosome map.

If we make the assumption that loci contributing to responsivity are randomly distributed along the length of the chromosome then it would appear that for the autosomes the distribution of these loci is constant per map unit.

We have already mentioned that phenocopy susceptibility was negligible in the Ore-R experiment. Because of this low responsiveness it was not feasible to explore the distribution of susceptible alleles further.

Gene Behavior

In the next part of the work, comparisons of phenocopy susceptibility were made utilizing chromosomes derived from the laboratory stocks under consideration. Because the strains from which the chromosomes were derived showed differences in phenocopy susceptibility and because the strains have been isolated breeding populations long enough for genetic drift to have occurred, it was hoped that genetic differences in the control of phenocopy responses might have developed and that these differences would be detectable. Except in the case of e¹¹ strain we have no evidence that the laboratory strains are genetically homogeneous with respect to phenocopy susceptibility and therefore may not suggest that the derived chromosomes are representative of the populations from which they were obtained.

In the three experiments where second and third chromosomes derived from different stocks were compared to their respective F_1 's for phenocopy susceptibility, it was consistently found that single heterozygotes (for example genotypes in which the second chromosome is heterozygous and the third chromosome homozygous) had a lower phenocopy susceptibility than their homozygous parents. Heterozygote superiority was also found in earlier experiments in which crosses were made between laboratory stock populations (Table 4).

One explanation of heterozygote superiority is that there are loci at which the heterozygote is superior to either homozygote. This explanation is considered unlikely as single locus heterozygote advantage has never been unequivocally established for any locus in Drosophila (Merrel, 1965). The explanation of this phenomenon which we favor is that genes responsible for phenocopy responsiveness are recessive and that these recessive loci are different in chromosomes from different strains, thus, in the heterozygote, recessive alleles from both parents are masked by dominant alleles which appear to lower phenocopy response. This interpretation does not explain the behavior of the double heterozygote in the e^{11} - wh_{sn}^3 experiment which will be considered below. Returning to the explanation of heterozygote superiority of single heterozygotes of either the second or third chromosome, a minimal number of loci controlling phenocopy susceptibility may be

postulated as two for the second and two for the third chromosome.

The necessity for postulating at least two loci may be seen from comparing the expectations of the one versus the two locus model in Fig.

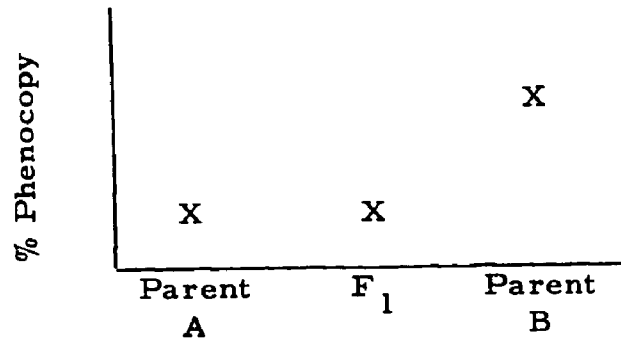
5. In a one locus model the F_1 would respond the same as the parent with the dominant gene whereas in the two locus model the F_1 response may be lower than either parent since each parent has deleterious alleles which are masked in the hybrid. A critical test of the two loci per chromosome hypothesis would involve recombination experiments.

Another interesting finding in our experiments concerns the existence of interactions between second and third chromosomes in the e^{11} -Ore-R and e^{11} -w^hsn³ experiments as well as a similar indication in the e^{11} -w^{bl} experiment. As pointed out previously, the double heterozygotes in the e^{11} -Ore-R and e^{11} -w^{bl} experiments were not appreciably less phenocopy susceptible than the single heterozygotes whereas in the e^{11} -w^hsn³ experiment the double heterozygote actually appears to be more phenocopy susceptible than the single heterozygote

Fig. 5

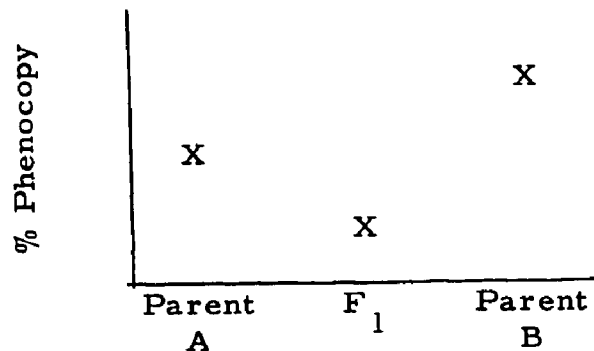
Interpretations of F_1 Phenocopy Responses

- 1) Expectations if genes of one parent are dominant to genes of the other parent



If an effective buffering allele in parent A masks a recessive allele in parent B, the phenocopy susceptibility of the F_1 should be the same as parent A

- 2) Expectations if both parents contain genes dominant at different loci



If an effective buffering dominant alleles in parent A masks a recessive allele in parent B and vice versa the phenocopy susceptibility of the F_1 should be lower than that of either parent

genotypes although generally less susceptible than their homozygous parents. Another way of stating this situation is that a given heterozygous chromosome (for example, a second chromosome) behaves differently on homozygous third chromosome backgrounds than on a heterozygous third chromosome background. In this case the double heterozygote has a reduced ability to achieve buffering of a developmental pathway. While most discussions of the genetic control of developmental homeostasis have stressed the significance of heterozygosity (Lerner, 1954), it has never been firmly established whether beneficial phenotypes result from heterozygosity per se or from the genetic contents of particular loci. The present work suggests that the gene action and the physiological effects of the heterozygous loci involved have to be understood before one can predict whether the sum of the heterozygous loci in an organism will be optimally beneficial. Once again, the dictum that the harmfulness, neutrality or utility of a particular gene depends on the genotype as a whole, is relevant (Dobzhansky and Spassky, 1968).

On the basis of several previous observations we may be able to probe the nature of the relative double heterozygote "inferiority" further. From the appearance of F_1 unresponsiveness relative to their homozygous parents we have suggested that different strains utilize different alleles to achieve buffering. It is entirely possible therefore that different strains are using different physiological methods to achieve

the same goal of homeostasis. In addition a given strain may itself have more than one buffering mechanism as suggested by the fact that single heterozygotes of either chromosome give substantial buffering. It goes without saying that within a strain the several mechanisms evolved for buffering would be complementary, that is they would act together harmoniously. The harmonious relationship among genes has been called coadaptation and evidence for it has been obtained from studies on heterosis in inversion heterozygotes (Dobzhansky, 1950), and the breakdown of heterosis in the F_2 generation of hybrids between populations from different geographical locations (Vethukov, 1953). Apparently in the present experiments, buffering capability was lost in the double heterozygote owing to the lack of coadaptation of the genes from the two different strains.

Other examples of "inferiority" double heterozygotes relative to their single heterozygotes are hard to find. For example in the prune-prune Killer system lethality will result only when the dominant prune Killer in homozygous or heterozygous conditions is combined with the sex linked prune allele in either the hemizygous or homozygous condition (Sturtevant, 1956). In other examples of two locus lethal interactions the interacting alleles must be hemizygous or homozygous as seen from the work on rosy-dor interactions (Lucchesi, 1968). In this study double heterozygotes were clearly more viable than the single

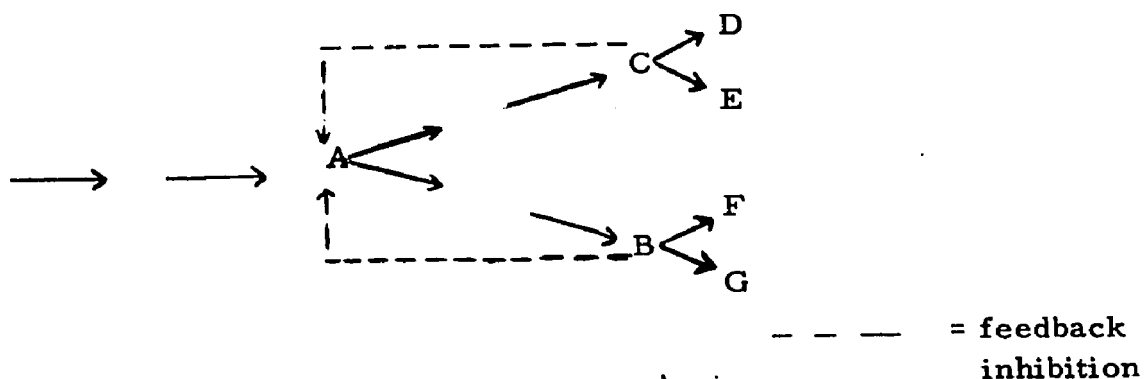
heterozygotes, contrary to the results which we obtained. An example of lethal interactions in hybrids may be seen in the work of Hollingshead (1930) who found a lethal allele in Crepis which only expressed itself in an interspecific cross. The situation is complicated by the fact that one of the species contained a chromosome complement of n equal to three while the other contained four different types of chromosomes. Thus, while the lethality may have arisen from the interaction of heterozygotes we cannot rule out the possibility that more complex gene balance problems are involved. Furthermore the system does not allow comparisons between single and double heterozygotes to be made. Kearsey and Kojima (1967) performed an experiment which gives more clearcut information concerning comparisons between single and double heterozygote performance. In their study, chromosomes were derived from inbred wild-type strains in a manner very similar to that used in our experiments. When second and third chromosomes derived from Samarkand and Swedish b strains were combined in all homozygous and heterozygous combinations and these genotypes were tested for percent hatchability, a dominant by dominant chromosome interaction was found. When the above study was performed on the Samarkand x chromosomal background the average hatchability of the four single heterozygotes was 98.3% with a range of 97.75% to 98.75% while the double heterozygote had an average hatchability rate of 95.75%. Here

we see another indication of double heterozygote "inferiority" relative to the single heterozygotes. When the same chromosomes were on a heterozygous or Swedish b x chromosome background the double heterozygote was the same as or superior to the single homozygotes. Having found at least one other example of apparent double heterozygote "inferiority" we may raise the possibility that this phenomenon may occur quite frequently in inter-strain crosses, but has simply gone unobserved.

A model for explaining this double heterozygote situation may be constructed if one assumes that each strain has a buffering mechanism which requires the same substrate at one point in the pathway but which subsequently diverges along different metabolic routes. With both mechanisms competing for the same substrate in the double heterozygote, there may not be sufficient substrate for either mechanism to work optimally. This model is elaborated in Fig. 7. In the model, regulation is seen to occur at the branch points of a pathway. Support for the idea that genetic control is exercised on the first step of a metabolic sequence comes from the work of Kacser (1963). Kacser has shown from basic kinetic considerations as well as experimentally, that intermediate steps in an unbranched part of a biochemical pathway have inherent homeostatic properties within extremely wide ranges of perturbation. To control the rate of synthesis along the pathway, regulation can only be exercised on the first and last steps of the sequence. Thus

Fig. 6

One Possible Model To Explain How Buffering Mechanisms
Might Interfere With One Another



Feedback inhibition of product C on the step preceding A constitutes the buffering mechanism in strain 1 and feedback inhibition of B on the same step constitutes the buffering mechanism of strain 2. Excess accumulation of products D, E, F or G would be harmful to the organism. At a sufficiently high concentration of C or B the feedback inhibition would be initiated but when both pathways are competing for A, B and C rarely attain sufficient concentrations to have an inhibitory effect. Thus products D, E, F, and G accumulate with consequent deleterious effects to the organism.

the only mutations which are likely to alter a phenotype are those affecting the first or last step.

Other schema may be advanced to explain harmonious or non-harmonious gene interactions. One line of thought is that a quantitative balance between different metabolites may be maintained with harmonious genes whereas nonharmonious groupings lead to metabolic disturbances. Evidence for the balance hypothesis was provided by Emerson (1948) who found that the heterocaryon between a sulfanilimide requiring strain and a suppressed sulfanilamide requiring strain grows better on minimal media than does either strain alone. Actually the model of metabolic regulation advanced in the preceding paragraph is an example of the kind of extensions of the gene balance idea which can be constructed.

In addition to its implications for the mechanisms of the metabolic control of pathways in development, the presence of interaction is also relevant to the main hypotheses concerning the genetic basis of phenocopy susceptibility.

The interaction between second and third chromosomes casts light on the main hypotheses concerning the genetic basis of phenocopy susceptibility. We briefly mentioned in the introduction — that Landauer (1957) believes that "...sporadic defects as well as experimental phenocopies are the results of events through which ordi-

narily hidden weaknesses of developmental equilibria become manifest and that these weaknesses have a definite, if complex genetic basis" while Goldschmidt (1957 a) states that, "All this may be understood under a genetic and developmental scheme as developed by Landauer But a group of further facts point strongly to the idea that quantitative and qualitative specificities of phenocopic action in different stocks, the specific effects upon penetrance and the relations to so-called spontaneous variants, are all based upon the presence everywhere of an array of subthreshold mutants. . . ." From the present study it becomes evident that in addition to individual chromosomal contributions to phenocopy response, in certain cases there is also an additional contribution through interaction of the second and third chromosomes. Interaction implies that one gene can modify the activity of another; this constitutes a type of regulation. This type of emergent regulatory behavior is supporting evidence for Landauer's view that the genetic basis of phenocopy susceptibility resides in ineffective buffering alleles. Such interaction effects may explain why Goldschmidt and Piternick (1957 a) were unable to select for high reactive lines in some of their experiments with sodium tetraborate. We may then conclude that phenocopy phenomena may be utilized fruitfully not only for the purpose of discovering isoalleles but also for the elucidation of the type of gene action responsible for the regulation of developmental homeostasis.

We may now make some general statements concerning the nature and significance of the variability encountered in the experimentation on glucosamine phenocopy susceptibility. We found both environmental (e. g., day to day) variation within a given experiment as well as genetic variation in the phenocopy response of different genotypes. The genetic differences among the genotypes examined would not have been uncovered under normal conditions without the aid of an external disturbing influence, in this case, glucosamine hydrochloride. One conclusion reached was that different strains utilized different alleles to buffer the aminosugar pathway. The wide differences we found in the genetic control of phenocopy in different strains is probably due to the small number of strains considered in the first experiments and the small sample of chromosomes tested in the experiments of the Latin-square design. Had we sampled more widely of the genes available in Drosophila we undoubtedly would have occasionally found certain strains using largely the same alleles in the buffering system. Considering however the rapidly expanding body of knowledge on genetic variation in populations, the heterogeneity observed is hardly surprising. The extensive work of Dobzhansky in the genus Drosophila has amply borne out this contention with respect to genes controlling viability while Lewontin and Hubby (1966) have demonstrated an almost unprecedented amount of polymorphism in the electrophoretic behavior of enzymes in

D. pseudoobscura. The significance of genetic polymorphism in general and heterozygosity in particular seems to stem in part from the increased homeostatic ability of heterozygous individuals. In our data we have presented evidence that heterozygosity, up to a point is associated with effective buffering. When heterozygosity was extended from one chromosome to two chromosomes, the buffering ability of the genotype was seen to decrease in one instance. Thus optimal genetic buffering systems appear to depend on heterozygosity coupled with mutually compatible alleles (Dobzhansky and Wallace, 1953).

Considering the components of environmental variation, day to day variation (run component) appeared to be most important. In the experiment among e¹¹ chromosomes as well in those involving comparisons between chromosomes derived from e¹¹ and Ore-R and those derived from e¹¹ and w^{bl} day to day variation was significant. Among the variables which might produce the day to day differences such factors as the condition of the parents, differences in larval food media, room temperature while collecting larvae and placing them on paper pulp and microflora on the paper pulp may have played a role. These variables did not produce detectable differences in replicates made on the same day, however. The presence of significant day to day variation indicates that uncontrolled environmental variables can have considerable effect on phenotype susceptibility. Thus unless genotypes are screened on the same day

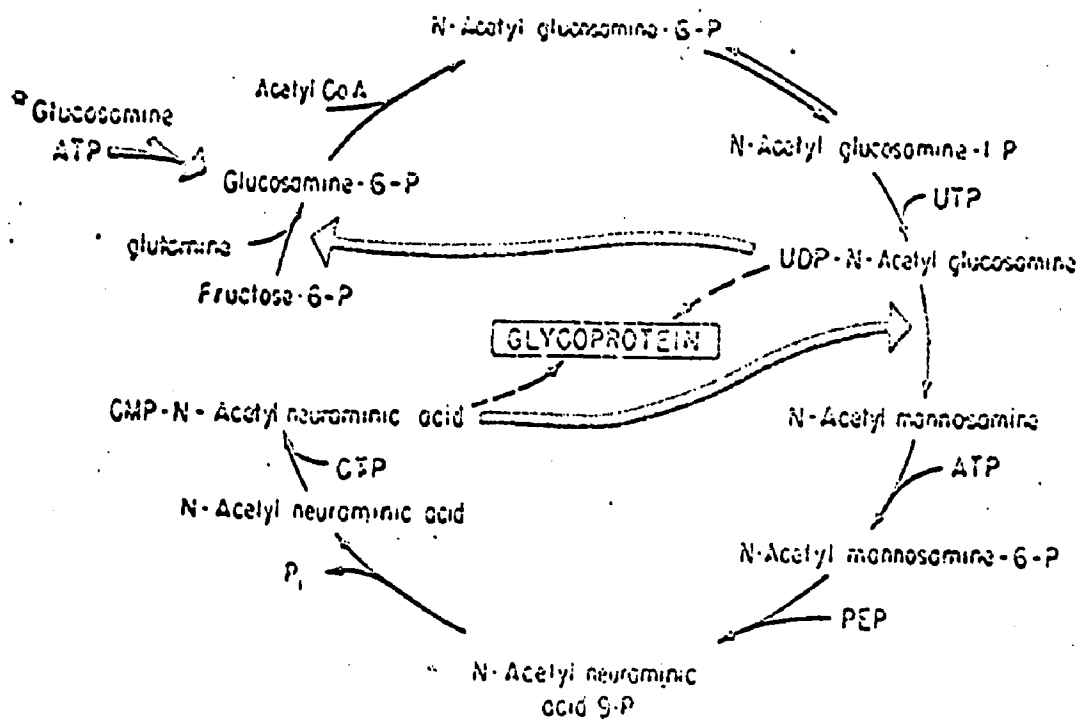
they cannot be compared so far as phenocopy susceptibility is concerned unless statistical methods are used to partition variation into environmental and genetic components. An understanding of how environmental influences affect the phenocopy susceptible system will have to await elucidation of the physiology of the system.

Mechanism of Glucosamine Effect

If we specifically consider the phenocopy response to glucosamine it might be useful to correlate what has been revealed by our experiments to what is known of glucosamine metabolism in higher organisms. Figure 8 is a reproduction of a chart of glucosamine metabolism found in Kornfeld and Kornfeld (1965) which will be used here for orientation. Fristrom (1964, 1968) found glucosamine-6-phosphate, N-acetylglucosamine-6-phosphate, N-acetylglucosamine-1-phosphate and uridine diphospho-N-acetylglucosamine as well as N-acetyl galactosamine. uridine diphospho-N-acetylgalactosamine uridine diphospho glucose and mannosamine in Drosophila melanogaster. With respect to metabolic control, in addition to the end product inhibition in rat liver found by Kornfeld and Kornfeld (1965) which is indicated by the broad arrows in Figure 8, several other regulating mechanisms have been shown to occur in higher organisms as seen in Table 12. Furthermore, several possible shunts exist which would tend to deflect intermediates into

Fig. 7

Synthetic Aminosugar Pathways



Broad arrows indicate sites of feedback inhibition

Table 13

Control Mechanisms Operating on GA Pathway

Substrate	Enzyme Inhibited	Enzyme Induced Or Activated	Source	Organism
UDPNAGA	glutamine-F6P transaminase		Kornfeld, 1964	Rat
UDP glucose	"		"	"
Glucose	GA kinase		McGarrahan & Maley, 1962	"
NAGA6P		Activates GA deaminase	Comb & Roseman, 1958	hog
GA	glucosamine			
GA6P	glucosamine 6-phosphate			
NAGA	N-acetyl glucosamine			
NAGA6P	N-acetyl glucosamine 6-phosphate			
UDPNAGA	uridine diphospho N-acetylglucosamine			
F6P	fructose 6-phosphate			

other pathways. For example glucosamine-6-phosphate might be deaminated and utilized in glycolysis. Likewise, deacetylation of N-acetyl glucosamine could occur. Considering the branched nature of these pathways as well as the considerable possibility for metabolic control, our interpretation of the possible significance of dominant by dominant gene interaction cannot be dismissed.

Up to this point we have been concerned with the phenocopy response to glucosamine as an indication of metabolic disorder but little attention has been given to the significance of the glucosamine pathway to the organism. The general products of this pathway are glycoproteins. These substances are found in insect integument and peritrophic membrane as a chitin-protein complex. The peritrophic membrane is a sheath secreted by specialized cells (PR cells) of the proventriculus, a midgut organ (Rizki, 1956). The sheath forms a tube which surrounds ingested food as well as excreta throughout the mid and hindgut. The intriguing possibility exists that excess glucosamine may be converted to insoluble chitin by the PR cells and secreted in the form of the peritrophic membrane. Utilizing C^{14} Glucosamine in autoradiographic experiments it has been shown that the PR cells incorporate label more heavily than other cells in the organism. Therefore, we cannot dismiss the possibility that the chief defect in susceptible individuals is their inability to remove excessive glucosamine by converting it into

peritrophic membrane. Another possibility which must not be overlooked is that resistant individuals are unable to absorb glucosamine through the gut epithelium. The investigation of a cellular mechanism for the buffering of the aminosugar pathway is all the more promising from the fact that strictly biochemical evidence suggests that different metabolic pools exist for the metabolism of glucosamine and N-acetylglucosamine (Fristrom 1965, 1968). Furthermore, attempts to establish metabolic regulation in *Drosophila* of the type found by Kornfeld et. al. (1965) in rat liver, have failed (Fristrom 1968).

In addition to chitin, other glycoproteins are known to be of importance in insect development. For instance, secretory substances used to cement the pupal case to substrata are known to contain glycoproteins. Of greater relevance to the crc phenocopy problem, however, is the role of glycoproteins as structural units in cell membranes. At least part of the function these molecules play in cell membranes concerns intercellular adhesivity.

The phenomenon of cell adhesivity is of obvious significance for the evolution of multicellularity. Not until Holtfreter's studies of 1939 was the significance of cell adhesivity in morphogenesis fully appreciated. Holtfreter's work consisted of combining explants of amphibian embryonic tissue in vitro. When early gastrula ectoderm and endoderm were cultured together the tissues adhered to one another at first but

in time separation occurred such that an ectodermal ball pinched off from the endoderm. This separation is reminiscent of normal development in which mesoderm eventually separates these tissues from one another. In fact, when explanted mesoderm was combined in culture with ectoderm and endoderm the mesoderm became sandwiched between the other layers. Additional studies showed that as a tissue differentiates its adhesive properties are altered. For example when ectoderm gave rise to neural tissue the neural tissue tended to sink into epidermis. This work provided evidence for considering morphogenetic movements as the result of the self ordering of embryonic tissues rather than as the consequence of physical pressures or other environmental factors associated with randomly migrating cells. Thus a unified explanation was provided for the myriad of cell and tissue movements associated with embryonic processes beginning with gastrulation and continuing through organogenesis. Since Holtfreter's work numerous studies have extended his findings to show that in addition to sorting out according to embryonic tissue affinities, individual cells may sort out according to histological origin. For instance, Moscona (1957) demonstrated that mixtures of dissociated liver cells from chick and mouse embryos were able to aggregate to form hepatic cords consisting of interspersed mouse and chick cells. When dissociated chick chondrogenic cells and mouse liver cells were combined, the two cell types sorted out,

cartilage cells forming central clusters with hepatic cells surrounding them. These and similar studies provide ample evidence of the intrinsic ability of cells to participate in specific cell-cell adhesion and provide further evidence for the importance of this phenomenon in histogenesis.

Problems of cell association have been studied in insects as well as in vertebrates. In the course of studying field properties in *Drosophila* imaginal discs, Hadorn et. al. (1959) demonstrated that partially disaggregated wing discs from different organisms will reaggregate when cultured in a larval host. After the larva has metamorphosed the tissue may be dissected out and is found to be a mosaic of aggregates from the different organisms which nevertheless may form integrated hair patterns. The nature of the cell affinities found have been investigated further. García-Bellido (1966 a, b) using similar techniques found that cell fragments will fit together according to where they belong in a predetermined pattern. When cells were transdetermined, let us say from leg cells to wing cells, the transdetermined wing cells would seek associations with other wing cells. Thus selective cell affinities are amply documented in insect tissue as well as vertebrate tissue. Despite the importance of the subject little work has been done to establish the metabolic basis of cell adhesion. An in vivo demonstration of the ability of glucosamine to alter cell adhesivity in Drosophila was provided by Rizki (1961) who showed that dietary glucosamine inhibits the lamellocyte to lamellocyte

adhesion in melanotic tumours of the larvae of the tu^W strain. These results indicate that the glycoprotein portion of cell membranes may play a decisive role in cell adhesion of *Drosophila*. They further suggest the possibility that ingested glucosamine may have far reaching effects on the organism for there is no reason to believe that the membrane metabolism disturbance found is confined to the lamellocytes. Indeed, Rizki has suggested the possibility that adhesion may be decreased in body cells after glucosamine treatment leading to the crc phenotype since the hydrostatic pressure which usually is sufficient to evert the cephalic structures may be ineffective owing to lack of cellular continuity. Fristrom (1965) however, was not able to confirm this hypothesis. Supporting evidence of the ability of glucosamine to alter cell adhesion is to be found in the studies of Garber (1962, 1963) who worked with dissociated embryonic chick cells in vitro.

At this time we are not in a position to say whether the control of the phenocopy response is exerted at the molecular level via enzyme regulation or at a secondary cell-cell level. The significance of this phenocopy system however lies in that analysis can probably be extended from a genetic to a biochemical and cellular level. Such a three part analysis might provide the first detailed model of a mechanism by which homeostasis is achieved in a developmental pathway.

CONCLUSIONS

Glucosamine is the first reported case of a specific endogenous metabolite which acts as a phenocopy agent. Upsetting the metabolic pattern of aminosugars by overloading the developing organism with glucosamine is a unique approach to the investigation of the general problem of genetic control of developmental homeostasis.

The following genetic properties of the glucosamine phenocopy control system were found:

a) the x chromosome does not appear to influence phenocopy susceptibility while phenocopy susceptibility was associated with both second and third chromosomes.

b) evidence for dominant gene action as well as epistasis was found in all experiments where comparisons were made between chromosomes derived from different stocks. While heterozygosity in either the second or third chromosome appeared to confer phenocopy tolerance the double heterozygote in the e¹¹ and w^h_{sn}³ combination appeared to be more phenocopy susceptible than the single heterozygotes. This indicates that phenocopy tolerance and thus developmental homeostasis in this system requires a balance of harmoniously interacting genes. The occurrence of epistatic regulatory genes in this study strongly supports Landauer's contention that experimental phenocopies result from a

genetically based weakness in developmental equilibria and not only, as Goldschmidt maintained, on arrays of sub-threshold mutants.

c) Phenocopy susceptible genes did not appear to be allelic in chromosomes derived from different laboratory strains.

Utilizing phenocopy phenomena in conjunction with the statistical and genetic techniques described we have uncovered hidden genetic variability in a system involving developmental regulation. As it appears that animals of different genetic constitutions utilize different alleles to achieve homeostasis, we may expect that these alleles act on different processes as well. Thus, the genotypes synthesized in the course of this work may provide stocks potentially useful for analyzing the component pathways culminating in the phenocopy response.

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APPENDIX
1A

Description of mutants in stocks used*

Symbol	Name	Chromosome: location	Description
cn	cinnabar	2:57.5	bright red eye
cn ²	cinnabar ²	2:right arm	bright red eye
Cy	Curly	2:6.1	curly wings
cp ^{txI}	dumpy-thoraxate of Ives	2:left arm	homozygous lethal
D ³	Dichaete ³	3:40.4	wings extended and elevated
e	ebony	3:70.7	black body
e"	ebony"	3:70.7	black body
h	hairy	3:26.5	extra hairs on scutellum
Me	Moire	3:19.2	shimmering eye
pr	purple	2:54.5	ruby eye color
ri	radius imcompletus	3:47.0	interrupted wing vein
S .	Star	2:1.3	small rough eyes
Sb	Stubble	3:58.2	shortened thickened bristles
Sp	Sternopleural	2:22.0	increased number of sternopleural
sn ³	singe ³	1:21.0	curved bristles and hairs
w ^{bl}	white-blood	1:1.5	yellow ruby eyes
w ^h	white-honey	1:1.5	honey colored eyes

APPENDIX 1A (continued)

Description of mutants in stocks used*

Linkage Relationships in j172 Stock

Chromosome	Right arm	Left arm
2nd	cn ²	S Sp
2nd	cn ²	dp ^{txI} Cy pr
3rd	h ri D ³	e
3rd	Me	ri Sb

* Information obtained from Lindsley and Grell (1967).

Key:

- N = number of zygotes examined
- q = fraction of zygotes showing phenocopy
- Tr = transformed value of q according to
arcsin of the square root of $q \times 100$
- H = heterozygote
- A = replication A
- B = replication B
- II = chromosome 2
- III = chromosome 3

APPENDIX 2A

Raw Data e"

Genotype
III



II

	H2O	GA	H2O	GA	H2O	GA
A	N 96 0.0 0.0	88 0.057 13.81	35 0.0 0.0	98 0.040 11.68	57 0.0 0.0	104 0.063 14.54
B	N 77 0.0 0.0	155 0.058 13.94	81 0.0 0.0	148 0.101 18.53	50 0.0 0.0	79 0.221 28.04
		Run 1		Run 2		Run 3
A	N 105 0.0 0.0	122 0.61 51.88	57 0.018 7.71	113 0.230 28.66	7 0.0 0.01	12 0.083 16.74
B	N 90 0.0 0.0	97 0.582 49.72	91 0.03 10.47	98 0.174 24.65	35 0.0 0.04	90 0.233 28.86
		Run 3		Run 1		Run 2

APPENDIX 2A (continued)

Raw Data e"

Genotype
III



II



	H2O	GA	H2O	GA	H2O	GA
A	101 0.0 0.0	130 0.285 32.27	110 0.0 0.0	87 0.411 39.87	34 0.029 9.81	83 0.410 39.82
B	83 0.01 5.74	94 0.605 51.06	60 0.09 5.44	141 0.529 46.66	63 0.016 7.27	92 0.315 34.14
		Run 2		Run 3		Run 1

APPENDIX 3A

Raw Data Ore-R

Genotype
III →

II

	H2O	GA	H2O	GA	H2O	GA
A	N 141	159	29	142	53	98
	q 0.007	0.0	0.0	0.0	0.0	0.011
B	Tr 4.80	0.0	0.01	0.0	0.0	6.02
	N 47	105	101	131	120	107
	q 0.0	0.019	0.0	0.031	0.0	0.009
	Tr 0.01	7.92	0.0	10.14	0.0	5.44
		Run 1		Run 2		Run 3
A	N 79	123	79	134	75	154
	q 0.013	0.024	0.0	0.008	0.013	0.013
B	Tr 6.55	8.91	0.0	5.13	6.55	6.55
	N 58	137	87	159	38	158
	q 0.0	0.015	0.0	0.025	0.0	0.051
	Tr 0.0	7.04	0.0	9.10	0.01	13.05
		Run 3		Run 1		Run 2

APPENDIX 3A (continued)

Raw Data Ore-R

Genotype $\longrightarrow$

III

II

	H2O	GA	H2O	GA	H2O	GA
A	N	99	96	48	172	158
	q	0.0	0.010	0.0	0.012	0.006
	Tr	0.0	5.74	0.01	6.29	4.44
B	N	146	219	81	193	131
	q	0.021	0.032	0.0	0.010	0.031
	Tr	8.33	10.31	0.0	5.74	10.14
		Run 2		Run 3		Run 1

APPENDIX 4A

Raw Data e'' - w^{bl}

Genotype
III $\longrightarrow$

II	e''		H		w^{bl}	
	H20	GA	H20	GA	H20	GA
A	N	80	113	150	96	115
	q	0.0	0.062	0.0	0.01	0.20
	Tr	0.0	14.42	0.0	5.74	26.56
B	N	75	148	112	100	125
	q	0.0	0.088	0.018	0.010	0.064
	Tr	0.0	17.26	7.71	5.74	14.65
Run 2						
A	N	28	112	136	57	142
	q	0.107	0.054	0.052	0.018	0.007
	Tr	19.09	13.44	13.18	7.71	4.80
B	N	70	150	130	36	108
	q	0.014	0.033	0.054	0.0	0.019
	Tr	6.80	10.47	13.44	0.57	7.92
Run 3						
Run 1						
Run 2						

APPENDIX 4A (continued)

Raw Data e"-w^{bl}

Genotype III	e"		H		w ^{bl}	
	H20	GA	H20	GA	H20	GA
A	N	19	53	36	67	139
	q	0.0	0.170	0.028	0.0	0.072
	Tr	0.57	24.35	9.63	0.0	15.56
B	N	25	87	12	84	100
	q	0.040	0.276	0.0	0.010	0.090
	Tr	11.54	31.69	0.081	10.94	17.76
		Run 1		Run 2		Run 3

APPENDIX 5A

Raw Data $e''-w^h sn^3$

Genotype III		e''		H		$w^h sn^3$	
II	A	H20	GA	H20	GA	H20	GA
e''	N	46	65	109	111	41	76
	q	0.022	0.134	0.0	0.054	0.0	0.145
	Tr	8.53	21.47	0.0	13.44	0.01	22.38
	N	73	132	49	95	81	123
	q	0.0	0.114	0.0	0.053	0.012	0.220
	Tr	0.0	19.73	0.01	13.31	6.29	27.97
		Run 1		Run 2		Run 3	
H	N	91	140	119	115	39	59
	q	0.011	0.007	0.017	0.061	0.0	0.093
	Tr	6.02	4.80	7.49	14.30	0.01	17.76
	N	84	136	59	92	8	25
	q	0.0	0.052	0.017	0.044	0.0	0.040
	Tr	0.0	13.18	7.49	12.11	0.03	11.54
		Run 3		Run 1		Run 2	

APPENDIX 5A (continued)

Raw Data $e'' - w^{h_{sn}3}$

Genotype III →		e''				H				w ^h sn ³			
		H20		GA		H20		GA		H20		GA	
II													
↓ w ^h sn ³	N	70	132	41	95	81	92	0.025	0.044	87	63	0.095	17.95
	A q	0.014	0.053	0.0	0.021	0.0	0.0	9.10	12.11	0.0	0.0	0.0	0.0
	Tr	6.80	13.31	0.01	8.33	10.47	6.29						
	N	78	122	0	21								
	B q	0.013	0.090	0.0	0.0								
	Tr	6.55	17.46		6.29								
		Run 2		Rune 3		Run 1							

Raw Data e"-Ore-R

II

III

		Ore-R		H		e"	
		H2O	GA	H2O	GA	H2O	GA
A	N	100	111	102	114	58	54
	q	0.0	0.018	0.0	0.018	0.0	0.259
	Tr	0.0	7.71	0.01	7.71	0.0	30.59
B	N	139	133	79	105	37	63
	q	0.0	0.098	0.0	0.0	0.027	0.413
	Tr	0.0	18.24	0.0	0.0	9.46	39.99
		Run 1		Run 2		Run 3	
A	N	106	111	124	117	90	127
	q	0.010	0.0	0.0	0.017	0.033	0.102
	Tr	5.74	0.0	0.0	7.49	10.47	18.63
B	N	105	133	110	155	96	128
	q	0.010	0.008	0.0	0.007	0.0	0.047
	Tr	5.74	5.13	0.0	4.80	0.01	12.52
		Run 3		Run 1		Run 2	

H

APPENDIX 6A (continued)

Raw Data e'' -Ore-R

Genotype III →		Ore-R				H		GA		e''	
II	→	H20	GA	H20	GA	H20	GA	H20	GA	H20	GA
A	N	102	126	59	81	121	100	0.025	0.400		
	q	0.0	0.087	0.0	0.0	0.0	0.0	9.10	39.23		
	Tr	0.0	17.16	0.01	0.0						
B	N	108	110	107	107	121	90	0.017	0.567		
	q	0.0	0.100	0.0	0.019	0.0	0.0	7.49	47.87		
	Tr	0.0	18.44	0.0	7.92						
		Run 2		Run 3		Run 1					

APPENDIX 7A

Proportion of Total Error Due to Binomial
Variance in e"-w^{bl} Experiment*

$$u = \frac{\frac{pq}{\bar{n}_h}}{\frac{pq}{\bar{n}_h} + \theta^2} = \frac{\text{Binomial Variance}}{\text{Total Variance}}$$

p is the average proportion of phenocopy in each cell

q is 1-p

$\bar{n}_h$ is the sum of the reciprocals of the number of larvae used in each observation

$$u = \frac{0.10738(0.9262)}{\frac{117.26}{9.75}} < 0.01$$

*Calculations based on proportions of phenocopy of glucosamine treated animals.

APPENDIX 8A

Cell code refers to data in Appendices 2A-6A. Each experiment has nine cells which are coded for purposes of cross-references as follows:

1	2	3
4	5	6
7	8	9

APPENDIX 8A (continued)

Experiment in Appendix 2A	cell number	replication	treatment	number of pupae crg	number of pupae affected	number of pupae normal	total number of pupae
1	A	GA	GA	5	6	77	88
			H2O	0	3	93	96
			GA	9	2	144	155
			H2O	0	2	75	77
2	A	GA	GA	4	0	94	98
			H2O	0	0	35	35
			GA	15	4	129	148
			H2O	0	0	81	81
3	A	GA	GA	23	9	72	104
			H2O	0	3	54	57
			GA	5	9	65	79
			H2O	0	0	50	50
4	A	GA	GA	71	12	39	122
			H2O	0	6	99	105
			GA	60	10	27	97
			H2O	0	3	87	90
5	A	GA	GA	26	26	61	113
			H2O	1	12	44	57
			GA	17	1	80	98
			H2O	3	9	79	91

APPENDIX 8A (continued)

Experiment in Appendix 2A	cell number	replication	treatment	number of		number of		total number of pupae
				pupae c/c	pupae affected	pupae normal		
6	A		GA	1	2	9	12	
			H2O	0	0	7		
	B		GA	21	3	66	90	
			H2O	0	1	34	35	
7	A		GA	37	10	83	130	
			H2O	1	3	97	101	
	B		GA	41	6	47	94	
			H2O	0	3	80	83	
8	A		GA	46	9	32	87	
			H2O	1	3	106	110	
	B		GA	58	10	73	141	
			H2O	0	0	60	60	
9	A		GA	34	19	30	83	
			H2O	1	4	29	34	
	B		GA	29	12	51	92	
			H2O	1	4	58	63	

APPENDIX 8A (continued)

Experiment in Appendix 3A	cell number	replication	treatment	number of pupae crc	number of pupae affected	number of pupae normal	total number of pupae
1	A	GA	GA	0	3	156	159
			H2O	1	1	139	141
	B	GA	GA	2	0	103	105
			H2O	0	0	47	47
2	A	GA	GA	0	0	142	142
			H2O	0	2	27	29
	B	GA	GA	4	1	126	131
			H2O	0	8	93	101
3	A	GA	GA	1	2	95	98
			H2O	0	5	48	53
	B	GA	GA	1	1	105	107
			H2O	0	6	114	120
4	A	GA	GA	3	3	117	123
			H2O	1	3	75	79
	B	GA	GA	2	2	133	137
			H2O	0	0	58	58
5	A	GA	GA	1	1	132	134
			H2O	0	1	78	79
	B	GA	GA	4	4	151	159
			H2O	0	3	84	87

APPENDIX 8A (continued)

Experiment in Appendix 3A	cell number	replication	treatment	number of		pupae affected	number of		total number of pupae
				pupae crc	pupae normal				
6	A	GA	H2O	2		5	147	154	
				1		3	71	75	
				8		18	132	158	
				0		6	32	38	
7	A	GA	H2O	1		0	95	96	
				0		0	99	99	
				7		8	204	219	
				3		4	139	146	
8	A	GA	H2O	2		2	168	172	
				0		2	48	48	
				2		2	189	193	
				0		4	77	81	
9	A	GA	H2O	1		6	151	158	
				1		2	105	108	
				4		2	125	131	
				2		6	145	153	

APPENDIX 8A (continued)

Experiment in Appendix 4A	cell number	replication	treatment	number of pupae		pupae affected	number of pupae normal	total number of pupae
				pupae crc				
1	A	GA	H2O	7	18	88	113	
				0	9	71	80	
				13	5	130	148	
				0	10	65	75	
2	A	GA	H2O	0	0	150	150	
				0	2	53	55	
				2	0	110	112	
				0	3	117	120	
3	A	GA	H2O	23	8	84	115	
				1	9	86	96	
				9	4	112	125	
				1	4	95	100	
4	A	GA	H2O	6	5	101	112	
				3	2	23	28	
				5	5	140	150	
				1	6	63	70	
5	A	GA	H2O	7	4	125	136	
				0	2	84	86	
				7	3	120	130	
				0	2	83	85	

APPENDIX 8A (continued)

Experiment in Appendix 4A	cell number	replication	treatment	number of		pupae affected	number of		total number of pupae
				pupae crc	pupae normal				
6		A	GA	1		0	141	142	
			H2O	1		55	57		
		B	GA	2		0	106	108	
			H2O	0		0	36	36	
7		A	GA	9		3	41	53	
			H2O	0		4	15	19	
		B	GA	24		4	59	87	
			H2O	1		1	23	25	
8		A	GA	6		2	95	103	
			H2O	1		1	34	36	
		B	GA	1		2	82	85	
			H2O	0		1	11	12	
9		A	GA	10		10	119	139	
			H2O	0		14	53	67	
		B	GA	9		2	89	100	
			H2O	3		7	74	84	

APPENDIX 8A (continued)

Experiment in Appendix 5A	cell number	replication	treatment	number of			total number of pupae
				pupae crc	pupae affected	pupae normal	
1	A	GA	H2O	9	5	51	65
				1	3	42	46
				15	4	113	132
				0	6	67	73
2	A	GA	H2O	6	1	104	111
				0	1	108	109
				5	1	89	95
				0	1	48	49
3	A	GA	H2O	11	2	63	76
				0	0	41	41
				27	9	87	123
				1	5	75	81
4	A	GA	H2O	1	0	139	140
				1	0	90	91
				7	4	125	136
				0	2	82	84
5	A	GA	H2O	7	2	106	115
				2	4	113	119
				0	4	88	92
				1	2	56	59

APPENDIX 8A (continued)

Experiment in Appendix 5A	cell number	replication	treatment	number of pupae		pupae affected	number of pupae		total number of pupae
				crc	normal		normal	pupae	
6		A	GA	5		0	54		59
			H2O	0		0	39		39
		B	GA	1		0	24		25
			H2O	0		1	7		8
7		A	GA	7		5	120		132
			H2O	1		2	67		70
		B	GA	11		7	104		122
			H2O	1		2	75		78
8		A	GA	2		4	89		95
			H2O	0		2	39		41
		B	GA	0		0	21		21
			H2O	0		0	0		0
9		A	GA	4		8	80		92
			H2O	2		6	73		81
		B	GA	6		0	57		63
			H2O	0		8	79		87

APPENDIX 8A (continued)

Experiment in Appendix 6A	cell number	replication	treatment	number of pupae		pupae affected	number of pupae		total number of pupae
				crc			normal		
1		A	GA	2		2	107		111
			H2O	0		0	100		100
		B	GA	13		1	119		133
			H2O	0		0	139		139
2		A	GA	2		0	112		114
			H2O	0		0	102		102
		B	GA	0		1	104		105
			H2O	0		2	77		79
3		A	GA	14		5	35		54
			H2O	0		1	57		58
		B	GA	26		6	31		63
			H2O	1		0	36		37
4		A	GA	0		0	111		111
			H2O	1		1	104		106
		B	GA	1		1	131		133
			H2O	1		5	99		105
5		A	GA	2		1	114		117
			H2O	0		2	122		124
		B	GA	1		0	151		155
			H2O	0		0	110		110

APPENDIX 8A (continued)

Experiment in Appendix 6A	cell number	replication	treatment	number of		pupae affected	number of		total number of pupae
				pupae crc	pupae normal				
6	6	A	GA	13		2	112	127	
			H2O	3		2	85	90	
		B	GA	6		7	115	128	
			H2O	0		1	95	96	
7	7	A	GA	11		0	115	126	
			H2O	0		1	101	102	
		B	GA	11		1	98	110	
			H2O	0		3	105	108	
8	8	A	GA	0		1	80	81	
			H2O	0		0	59	59	
		B	GA	2		1	104	107	
			H2O	0		2	105	107	
9	9	A	GA	40		12	48	100	
			H2O	3		1	117	121	
		B	GA	51		12	27	90	
			H2O	2		5	114	121	