A DEVELOPMENTAL ANALYSIS OF THE LETHAL MUTANT L(2)GL OF DROSOPHILA MELANOGASTER BASED ON CYTOPHOTOMETRIC DETERMINATION OF NUCLEAR DESOXYRIBONUCLEIC ACID (DNA) CONTENT[1]

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IN the work to follow, a particular technique of analytical cytology—relative quantitative determination of desoxyribonucleic acid (DNA) by employment of the Feulgen reaction and subsequent measurement of the absorption of the DNA-bound dye in a cytophotometer by the two wave length method—has been applied in an analysis of aberrations in development of the lethal mutant, *l(2)gl* of *Drosophila melanogaster*.

As the ring gland is conspicuously affected in this mutant, necessary background information relates to the action of humoral agents upon growth and metamorphosis, concerning which several excellent reviews have recently appeared (BODENSTEIN 1954, 1955; ETKIN 1955; WIGGLESWORTH 1954). Particular attention is called to the view expressed by WIGGLESWORTH (1954) that "the moulting hormone has a quantitative effect on growth and not merely a catalytic or triggering action". According to WIGGLESWORTH, there would be two major conditions for growth, the supply of hormone or nutritional agents in general and the individual capacities of the various organs for utilization of these factors. If one of the latter should be limited, its effect on the various organs would be "integrated" and "proportionate". Further reference will be made to this subject when issues are defined in subsequent paragraphs.

Information concerning Drosophila growth and metamorphosis in particular, including the influence of the ring gland, can be found in the following references: HADORN 1937; HADORN and NEEL 1938; SCHARRER and HADORN 1938; VOGT 1940, 1942a,b, 1943, 1946; BODENSTEIN 1943a,b, 1947; DEMEREC 1950; WIGGLESWORTH 1954; ETKIN 1955.

Chiefly through the efforts of HADORN, collaborators, and associates (HADORN 1937, 1948, 1951; SCHARRER and HADORN 1938; HADORN and NEEL 1938; HADORN and RIS 1939; HADORN and GLOOR 1942; GLOOR 1943; CHEN 1951) a considerable body of data and theory has been accumulated concerning this mutant. The contribution of these workers consists mainly of qualitative cytological description, semiquantitative description on an organ level, gross quantitative description and analysis on the level of the whole organism, and division of effects into primary and secondary categories on the basis of transplantation experiments. Specifically, it has been stated (HADORN 1948) that the larval integument, musculature, alimentary

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tract, tracheal system, somatic cells of male and female gonads, imaginal ring gland cells, and possibly the central nervous system are not affected by the lethal constitution and that the ring gland itself seems secondarily affected. Little or no attention has been paid to the effect of the ring gland on larval development, possibly through concentration on what is regarded as its more important effect on pupation. No theory or hypothesis has been formulated concerning the nature of the primary effect.

If one grants the premise that certain tissues are affected and certain ones are not, the difficulty of offering a satisfactory hypothesis of unitary gene action in this case seems at the present moment insurmountable, as this organism seems to be suffering not from a selective effect peculiar to certain tissues but from a basic defect in the growth process which should affect all tissues. It is conceivable, of course, that the cell economy of certain tissues (WAGNER and MITCHELL 1955, p. 395) might be such as to discount the primary effect; conversely, positive evidence that such an effect does exist in all tissues would seem to be almost ironclad security for the idea of a fundamental interference in the growth process common to all tissues and differing in degree of manifestation according to the varying cytoplasmic constitutions and thus would remove the greatest obstacle to the hypothesis of unitary gene action in this mutant.

Furthermore, the absence of any consideration of a possible effect of the ring gland on larval development seems a striking gap in the theory. The general conclusions that have been drawn concerning insect development and certain suggestive if not conclusive evidence in Drosophila itself seem to make it legitimate to inquire whether hormonal influence is not partially responsible for the larval pattern of damage in this mutant, either independently and alone or superimposed upon a primary effect. If the evidence does indeed support such a conclusion, there would be the satisfaction of incorporating ring gland influence on larval development into the theory of development of this particular mutant and thus making it consistent with the general theory of insect development.

If we refuse to accept the premise of an effect in some organs and not in others and if we exercise a little disciplined imagination in regard to the larval influence of the ring gland, there are two hypotheses that seem internally consistent and logically satisfying. These are as follows: (1) all tissues are affected, some by a primary effect plus a superimposed hormonal effect, and others by the hormonal effect acting independently and alone; and (2) all tissues are affected, by a hormonal effect generally superimposed upon a primary effect common to all tissues.

These hypotheses share the assumption that all tissues are affected, which remains to be proved. They can be separated on the basis of WIGGLESWORTH'S theory as to the quantitative and proportionate effect of hormonal action, previously emphasized. Hormonal influence should act in the direction of a proportionate effect among the organs affected by the hormone alone. Except in the event of the implausible coincidence of the cell economies of two or more organs being so adjusted that the primary action has the same quantitative as well as qualitative effect, the primary effect should differentiate the hormonal effect and result in a total disproportionate effect, unless the former is slight in comparison with the latter.

If, therefore, most of the organs show a proportionate effect, hypothesis number one will be indicated. If, on the other hand, the disproportionate effect predominates, then hypothesis number two will be preferred.

The main purpose of this investigation, therefore, will be to determine whether or not all organs of the *l(2)gl* mutant are affected by the lethal gene and to what extent they are affected relative to both each other and to their normal counterparts.

Additional questions that arise concern the biochemical nature of the primary effect, of the hormonal effect, and of the relationship between the two.

MATERIALS AND METHODS

The successful application of quantitative cytochemistry to a problem such as this requires rigid discipline. It has been necessary to exert exacting control upon what might be called five levels of operation: the fly management level, the oviposition and incubation level, the cytological level, the staining level, and the photometric level.

The stock *l(2)gl cn bw/Cy cn bw* was obtained from the Cold Spring Harbor laboratory, crossed with L^2/Cy from this laboratory, and the *lgl cn bw/Cy* flies separated. As can be readily seen, the lethal larva carries the alleles *cn bw* in homozygous condition, which produce, among other effects, colorless Malpighian tubules in contrast to the yellow tubules of the normal, the definitive identifying characteristic of the lethal larva. For a control, in addition to the normal heterozygote of the lethal strain, the wild type Stephenville stock was used, which has over 90 percent hatchability.

Eggs of the same age were obtained by shaking down the flies from a tall Stender dish into a petri dish upon saline moistened filter paper in the center of which is a small mound of food medium, so that they do not fall directly upon the food itself. The flies are sexed, and about 500 females used at one time. In this way a superfluity of eggs is produced in a laying period of 30 minutes. Between 20 and $20\frac{1}{2}$ hours after oviposition all premature larvae are removed; larvae hatching during next $2\frac{1}{2}$ hours are placed in petri dishes on the standard medium used in this laboratory. Incubation is carried out at 25°C plus or minus .5 degree in an incubator kept in a constant temperature room.

Carnoy fixation (60-30-10 of absolute alcohol, chloroform, and acetic acid) was used and gave good results. After fixation from two to three hours, larvae were washed for about 30 minutes in several changes of absolute alcohol, cleared in Terpineol over a period of about 24 hours, infiltrated in 50-53 degrees melting point Tissuemat for $1\frac{1}{2}$ hours with two changes, and embedded. Sections were cut at from 4 to 25 microns according to the size of the nuclei; and ribbon orientation was obtained by observation of sections dissolved in xylol under a 20× phase objective.

Feulgen cytophotometry has been used in a variety of ways, especially in the field of cytology but also in embryology in its cytological aspect, physiology, and pathology (SWIFT 1953), although it has not yet proved as useful in the field of genetics as its sister technique, qualitative cytology, has been. The work to follow represents the first application of cytophotometry to developmental genetics.

Quantitative use of the Feulgen reaction, at least on a relative basis, is well established (RIS and MIRSKY 1949; SWIFT 1953; PATAU and SWIFT 1953; LESSLER 1953;

LEUCHTENBERGER 1954; NOVIKOFF 1955; POLLISTER and ORNSTEIN 1955). The photometric difficulties involved in the present investigation, chiefly irregularity of shape and inhomogeneity of distribution, have been met adequately by the two wave length method developed independently by ORNSTEIN (1952) and PATAU (1952), but in more usable form by the latter.

A rough check with a polarizing microscope showed no evidence of orientation in the material measured.

After hydrolysis in normal HCl at 60°C for 13 minutes, determined as the maximum period, slides were stained for two hours in Schiff reagent prepared according to the method of STOWELL (1945). Uniformity of staining procedure was preserved meticulously throughout the course of the work.

The refractive index of the tissue used in these measurements was determined by the method of GROAT (1941) to be between 1.530 and 1.535, which is rather closely matched by HSR (Harleco synthetic resin) mounting medium with a refractive index of 1.5202 in solution and 1.5390 as the dry resin (LILLE, ZIRKLE, DEMPSEY, and GRECO 1953).

As a check for nonspecific staining a "blank" was run, identical with test slides except for the omission of the acid hydrolysis prior to use of the Schiff reagent. This "blank" showed no coloration.

In order to measure nuclei with a light concentration of DNA, 570 millimicrons, at the absorption peak of the tissue, was chosen as the major wave length, according to the method of PATAU (1952), so that the minor wave length would have the greatest possible extinction. As the minor wave length 602 millimicrons was selected after it was found that this wave length had one half the extinction of the major in measurements between 20 and 60 percent transmission of homogeneous portions of several different types of nuclei, including brain tissue, convenient for use because readily available and consisting of multiple, small diploid nuclei, which taken together form a homogeneous area. The wave length ratio was checked during the course of the work in a number of different tissues on different slides and found to remain essentially constant.

The apparatus used here is modeled along lines developed by POLLISTER (1952) and POLLISTER and ORNSTEIN (1955). For description see KASTEN (1956). Standard tests, such as test of phototube for linear response to area of image measured and check of readings made through neutral density filters with those made in a Beckman spectrophotometer (SWIFT 1950; POLLISTER 1952) were made when the equipment was first set up by the writer and subsequently by KASTEN (1956). The linear response test has been repeated during the course of this work. Critical illumination (POLLISTER and ORNSTEIN 1955) and monochromator slit widths of .30 to .40 millimeters corresponding to band widths of 2.0 to 2.6 millimicrons have been used.

Precision in galvanometer readings is essential. The practice followed has been to make two sets of readings at each wave length, the blank immediately following the test, and, if the differences of these two pairs were more than one galvanometer point apart, to make one or more repeat readings until two were obtained sufficiently close together.

A four millimeter Spencer apochromat of N.A. 0.95 was used with a 3× ocular in

the microscope. At the plane of the phototube a 3× ocular served to locate the nucleus desired in the field of measurement, the diameter of which was determined by means of a micrometer scale highly magnified in a 30× ocular. A Lovins microslide field finder was used to record the coordinates of each nucleus measured, after the area had been mapped by camera lucida. Additional adjustments include the use of a low condenser aperture of N.A. 0.25 and restriction of the total illuminated field by means of the slit controls. Calculations were made according to the method of PATAU (1952).

Test measurements were made on 100 rat liver nuclei taken from six slides, three of which were stained on one date and three on another. Results of these, on file with the Editors of GENETICS, indicate that the Feulgen stain as carried out in this laboratory is reproducible from slide to slide to within ten percent or less, with the exception of the nuclei on one slide, which were about 15 percent off the overall mean. The results further show that the method, as practised in this laboratory, yields an accuracy in measurements of nuclei in the same individual and on the same slide represented by a coefficient of variation of from eight to ten percent. This could probably be reduced by taking the average of two or more replications.

Also on file with the Editors are the results of repeat measurements on eleven salivary gland nuclei of two hour prepupae, Stephenville wild type, *Drosophila melanogaster*, the field area being changed between the first and second measurements of each nucleus. These show an average reproducibility of less than five percent variation.

RESULTS

Quantitative

It was considered necessary to analyze a sufficient number of tissues to establish whether or not the effect is a general one, common to all organs. Organs analyzed quantitatively include the salivary gland, ring gland, corpus allatum, caeca, stomach, fat body, muscle, and epidermis; the effect on the tracheal system and brain is described qualitatively. Of these, as previously mentioned, at least five have been exempted from any effect of the lethal constitution (HADORN 1948). The only conspicuous omission is the gonads, which have not been included because DNA synthesis preliminary to meiosis would have confused the issue. Unless otherwise stated, growth is described in terms of the DNA content of the nucleus as a quantitative index.

An analysis was begun at the earliest possible stage and the course of the lethal effect charted at major intervals thereafter. First measurements were made on the 72-hour larva, as this is the earliest point at which the lethal can be separated from the normal; but, since measurements at this stage suffer from the disadvantage of a light concentration of DNA, only enough organs were examined to establish that the lethal effect is present at that period. The 120-hour lethal larva and the normal two hour prepupa, representing comparable stages, are the latest periods at which the normal larva can be analyzed and were chosen primarily for measurements on the salivary gland at or near the end point of larval development; but, for most organs, this is not a favorable stage for measurement since histolysis is beginning to cause

alteration or dissolution and in some instances this might lead to variability in staining. The 96-hour stage is a logical mid-point between the 72 and the 120-hour stages. Complete measurements on all organs examined quantitatively were made at this period and used as the basis for the main conclusions, as comparisons of the 96 and 120-hour measurements on salivary gland and ring gland showed that no advantage would be gained by analyzing at a period between 96 and 120 hours. Although growth continues in both lethal and normal until about 120 hours, the lethal effect stands fully revealed in the 96-hour larva.

For each category of organ effect, measurements were made on 15 to 20 or more nuclei, more than adequate according to the method used, drawn from a minimum of from three to five individuals. All nuclei were chosen at random when there was a choice except for the precaution that, when the tissue appeared in several parts of the larva, the regional location used was the same in all comparisons. Except in a very few instances when there was a legitimate reason for so doing, no measurements have been discarded, and no remeasurements have been made, since consistency would require that all, if any, nuclei be remeasured.

A comparison of DNA content of nuclei from ring gland and salivary gland in the Stephenville wild type and *lgl/Cy* heterozygote larvae shows no distinguishable difference. Whatever effect there is, if any, is apparently below the threshold necessary to produce an analyzable effect on this level.

Both growing and mature larvae show great uniformity. Whatever variation there is can be attributed to individual differences or to chance in the selection of nuclei, an issue that could not be resolved without a great deal more data. Whatever sex differences there are, if any, cannot be distinguished beneath the general overlay of varia-

TABLE 1

Determination of diploid and polytene class values from metaphase brain nuclei in two hour prepupa, Stephenville wild type, Drosophila melanogaster

No. nuclei*	Mean	Coefficient of variation	Diploid value
17	4.56	25.7%	2.28

Polytene class values

Class	Low	Mean	High
2 C	1.69	2.28	2.87
4 C	3.38	4.56	5.74
8 C	6.76	9.13	11.5
16 C	13.5	18.3	23.0
32 C	27.0	36.5	46.0
64 C	54.0	73.0	92.0
128 C	108	146	184
256 C	216	292	368
512 C	432	584	736
1024 C	864	1168	1472

* Average value per nucleus from measured field of from three to seven nuclei-total of 17 separate fields.

tion from experimental error and other causes. The fact of great uniformity, however, in speed of development and at maturity, does stand out.

Relative DNA values obtained have been divided into polytene classes based on measurements of diploid brain nuclei as given in table 1. In figures 1 through 4 and table 2 can be seen the degree of polyteny attained by the various types of nuclei. There is evident here a trend towards a slight reduction of class values below those in table 1. From table 2 it is apparent that the coefficients of variation obtained in measurements on Drosophila tissues range from 8.5 to 21 percent, with an average of about 15 percent or somewhat less, in contrast with the eight to ten percent variation obtained by the same method on rat liver nuclei. From table 3, relating particularly to the salivary gland, it is evident that the variation in the DNA content of nuclei of the same individual, when measurements are made on the same slide, ranges from 6.6 to 20.3 percent.

Comparison of lethal development with normal can be made by reference to figures 1 through 4 and table 4. On the whole, the picture of development that can be visualized from the statistics in terms of the DNA content of the polytene nucleus as a quantitative index of growth is coherent and intelligible. The lethal effect is quite evident at the 72-hour stage, reaches its peak at the 96-hour stage and continues

TABLE 2

Significant means and coefficients of variation of relative amount of DNA-Feulgen in nuclei of tissues in composite normal and lethal larvae of Drosophila melanogaster

Tissue	Normal			Lethal		
	Class and no. nuclei	Mean	C.V.	Class and no. nuclei	Mean	C.V.
72 hours†						
Salivary gland	256 C(12)	272	13.1	128 C(10)	131	14.0
Ring gland	32 C(18)	40.7	14.5	8 C(10)	9.44	13.2
96 hours†						
Salivary gland	256 C(13)	250	16.2	64 C(10)	63.7	12.9
	512 C(24)	471	16.0	128 C(13)	104	17.4
Ring gland	64 C(13)	65.4	11.0	32 C(13)	36.5	14.2
Corpus allatum	32 C(9)	30.9	13.3	16 C(9)	13.9	9.92
Caeca	128 C(16)	125	13.0	64 C(13)	69.8	11.8
Stomach	128 C(14)	134	21.0	128 C(9)	105	14.8
Fat body	64 C(14)	68.8	19.3	32 C(12)	37.0	21.0
Muscle	64 C(12)	57.9	18.0	16 C(11)	19.4	12.3
Epidermis	64 C(11)	66.1	14.1	16 C(9)	20.3	13.8
120 hours†*						
Salivary gland	256 C(10)	327	13.0	64 C(9)	66.4	15.8
	512 C(23)	515	17.4	128 C(5)	179	—
	1024 C(18)	963	15.8	256 C(5)	246	—
Ring gland	64 C(11)	55.6	8.61	32 C(13)	29.0	15.2

Polytene classes from calculated diploid value in table 1.

† Age in hours after oviposition.

* Two hour prepupa for normal

TABLE 3

Coefficients of variation of relative amount of DNA-Feulgen in nuclei of salivary gland in individual and composite larvae, Stephenville wild type, Drosophila melanogaster, *96 and 120 hours after oviposition*

Individual

96 hours				120 hours*			
No. individuals	Polytene class†	No. nuclei	Coefficient of variation	No. individuals	Polytene class†	No. nuclei	Coefficient of variation
1	512 C	11	13.8%	1	256 C	8	8.3%
2	256 C	9	6.6%	1	512 C	15	20.3%
2	512 C	6	18.2%	3	1024 C	7	11.3%
3	512 C	8	17.0%				
Composite							
	256 C	13	16.2%		256 C	10	13.0%
	512 C	24	16.0%		512 C	23	17.4%
					1024 C	18	15.8%

* Two hour prepupa

† Polytene class from table 1.

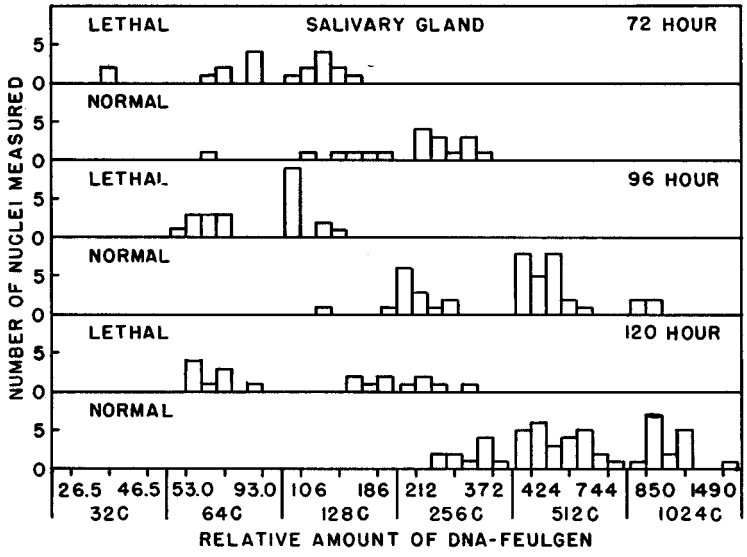

FIGURE 1.—Relative amount of DNA-Feulgen in individual nuclei of salivary gland in wild type Stephenville stock and lethal, *lgl/lgl*, larvae of *Drosophila melanogaster* at 72, 96, and 120 hours after oviposition. Abscissa divisions represent polytene classes from Table 1 and are discontinuous.

thereafter in about the same proportion. The salivary gland and the ring gland may be taken to epitomize the situation. It is apparent that the normal ring gland develops faster than the lethal since, at 72 hours, the latter is only about one third the former. By the time the 96-hour stage is reached, this disparity is somewhat reduced, although

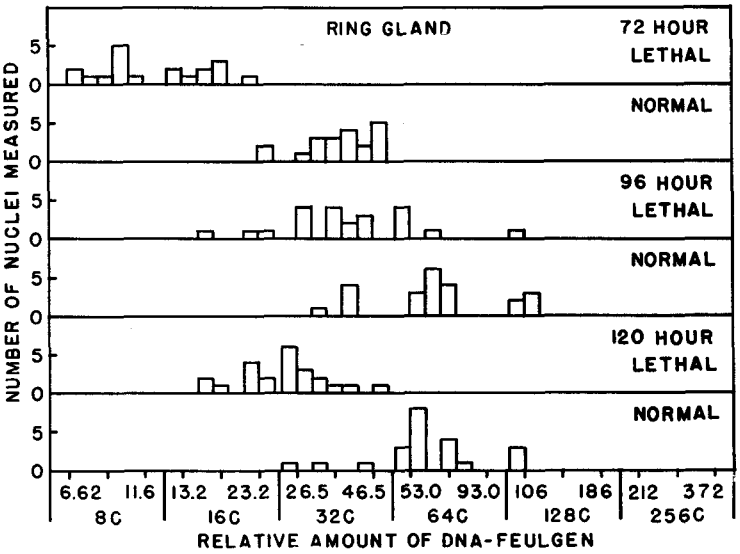

FIGURE 2.—Relative amount of DNA-Feulgen in individual nuclei of ring gland in wild type Stephenville stock and lethal, *lgl/lgl*, larvae of *Drosophila melanogaster* at 72, 96, and 120 hours after oviposition. Abscissa divisions represent polytene classes from Table 1 and are discontinuous.

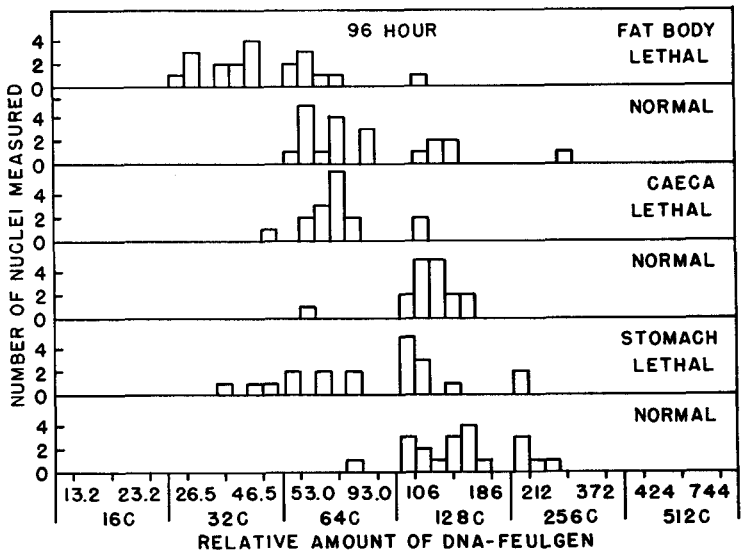

FIGURE 3.—Relative amount of DNA-Feulgen in individual nuclei of fat body, caeca, and stomach in wild type Stephenville stock and lethal, *lgl/lgl*, larvae of *Drosophila melanogaster* 96 hours after oviposition. Abscissa divisions represent polytene classes from Table 1 and are discontinuous.

the lethal ring gland never reaches full growth, and even displays some signs of degeneration indicated by the lower value at the 120-hour period. The course of development of normal and lethal salivary glands seems causally related to that of the ring gland. The normal salivary gland, for instance, develops rapidly from 72 to 96

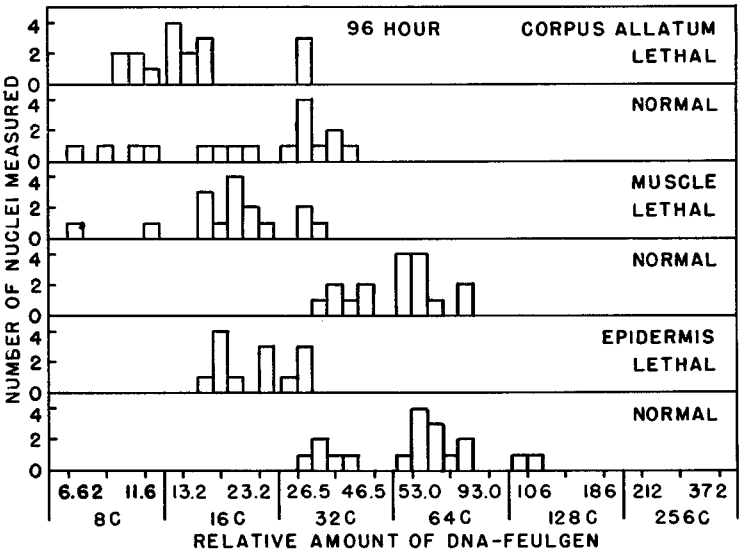

FIGURE 4.—Relative amount of DNA-Feulgen in individual nuclei of corpus allatum, muscle, and epidermis in wild type Stephenville stock and lethal, *lgl/lgl*, larvae of *Drosophila melanogaster* 96 hours after oviposition. Abscissa divisions represent polytene classes from Table 1 and are discontinuous.

TABLE 4

Lethal percent of normal based on relative amount of DNA-Feulgen in nuclei of tissues in composite normal and lethal larvae of Drosophila melanogaster

Tissue	Normal		Lethal		Lethal % normal
	No. nuclei	Average nuclear value	No. nuclei	Average nuclear value	
72 hours†					
Salivary gland	19	238	19	102	42.9
Ring gland	20	39.1	19	13.0	33.2
96 hours†					
Salivary gland	41	440	23	86.7	19.7
Ring gland	23	68.4	22	40.9	59.8
Corpus allatum	17	23.2	17	15.4	66.4
Caeca	17	121	16	73.9	61.1
Stomach	20	150	20	91.9	61.3
Fat body	20	96.1	20	48.1	50.1
Muscle	18	51.4	16	20.1	39.1
Epidermis	18	61.4	13	22.5	36.6
120 hours†*					
Salivary gland	51	636	19	143	22.5
Ring gland	22	62.2	23	26.6	42.8

* Two hour prepupa.

† Age in hours after oviposition.

hours as well as from 96 to 120, consonant with the early development of the normal ring gland; the lethal salivary gland, on the other hand, shows little increase until after 96 hours, at which time the lethal ring gland has, to some extent, "caught up" with the normal. Both the normal and lethal corpus allatum show a decrease in DNA at the 120-hour period, when its functional activity is over, as does the normal ring gland, to a slight extent, but the difference is not regarded as significant in the question of physiological variation of DNA with function.

As can be seen in table 4, after wide latitude has been allowed for the effect of experimental error on the percentages, four, or possibly five, different categories of

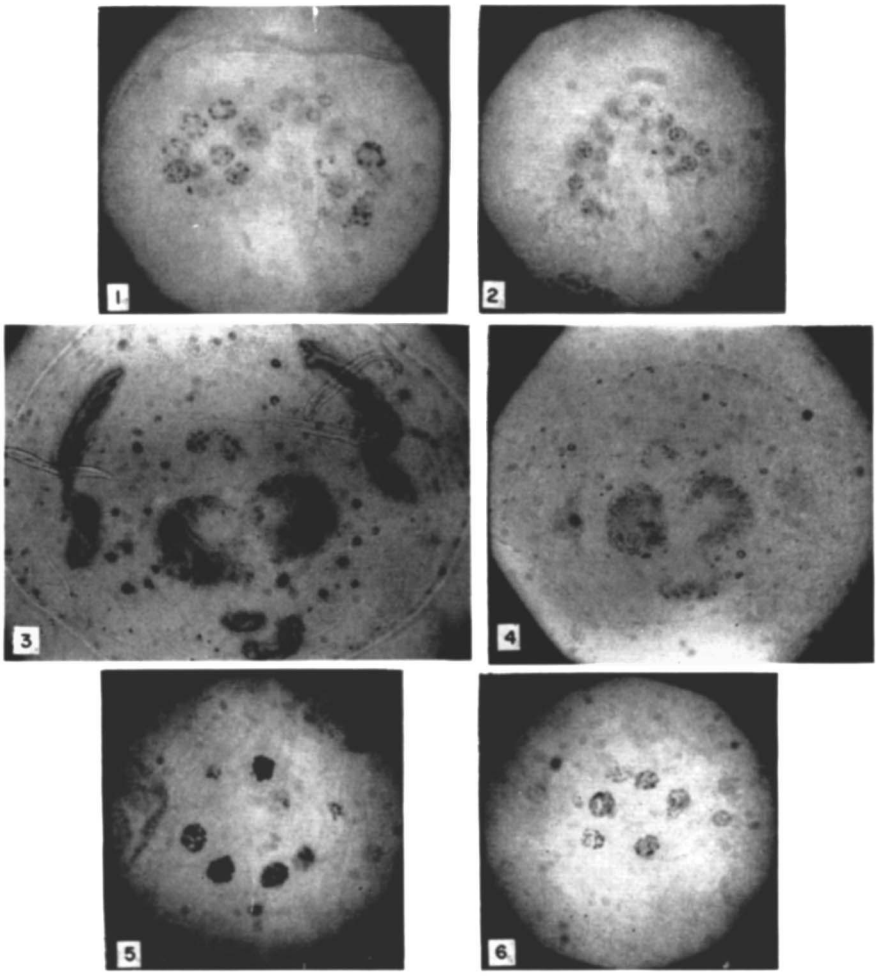

PLATE I, (1) through (6). Cross sections of Stephenville wild type and lethal, *lgl/lgl*, larvae of *Drosophila melanogaster*. Carnoy fixation. Feulgen stain. (1) Normal ring gland, 96 hours, ×600. (2) Lethal ring gland, 96 hours, ×600. (3) Normal brain area, 96 hours, including ring gland of (1), ×125. (4) Lethal brain area, 96 hours, including ring gland of (2), ×125. (5) Normal salivary gland, 2 hour prepupa, ×300. (6) Lethal salivary gland, 120 hours, ×300.

effect on four or five organs or groups of organs can be distinguished: the ring gland, together with the corpus allatum, which as sources of the hormones, must be placed in a category by themselves; the salivary gland; muscle and epidermis; stomach and caeca; and possibly the fat body. One cannot escape, however, the striking correlation between the range of development of the ring gland from 33 percent of normal at 72 hours to about 60 percent at 96 and the range of effect on other organs, with the exception of the salivary gland, from about 35 to 65 percent. Only the salivary gland exhibits a differential effect beyond these limits.

Qualitative

Two tissues, not analyzed quantitatively, show a distinct qualitative effect. The tracheal system, as seen in the spiracle region, displays effects on cytoplasmic and nuclear volume similar to those found in other organs and is therefore to be placed in the category of organs affected by the lethal mutation. Comparison of (3) and (4) in Plate I shows that the brain of the normal larva is both larger in area and heavier in DNA concentration than that of the lethal.

Within the limits of accuracy set by qualitative inspection of cross sections, one can say that the cytoplasm in all organs except the salivary gland shows about the same degree of reduction as the nuclei. The situation in the salivary gland is shown in (5) and (6) of the plate. Although the lumen in the lethal gland is larger than in the normal, the lethal cytoplasm appears reduced only about 50 percent, which compares favorably with the approximate 50 percent reduction of the whole gland previously found (HADORN 1948). In contrast with the cytoplasmic morphology, the lethal nuclei, in terms of DNA, are much more drastically reduced. The disproportionate character of the reduction is evident at 96 hours but most marked at 120, the stage of the salivary gland photographs. The normal nuclei in (5) range from about 1000 to 1500 in relative DNA values; while the lethal nuclei in (6) average about 165. The fact that the cytoplasm does not appear to be correspondingly reduced assumes considerable importance in regard to the nature of the primary effect.

DISCUSSION

It is necessary to omit discussion of polyteny and variation of DNA content in Drosophila nuclei because of limitations of space and in order to preserve unity of theme, which is the significance of DNA quantitation in explaining the developmental failure of *l(2)gl*.

Since, unequivocally, all organs examined, quantitatively and qualitatively, are affected, it seems reasonable to conclude that the effect, direct or indirect, of the lethal mutation is ubiquitous.

The known effect of the prothoracic and corpus allatum hormones on insect larval development in general, the failure of the ring gland in this mutant to achieve normal development, the demonstrated deficiency of the hormone in pupation, the ubiquitous effect on all organs, and the correlation of the effect on most organs with that on the ring gland itself, all taken together, furnish sufficient circumstantial evidence beyond reasonable doubt to incriminate the ring gland in retardation of larval development as

well as in failure of pupation. This is the logical explanation for the fact that the ovary shows improved growth when transplanted to a normal host (HADORN 1951).

The predominantly differential character of the effect would seem to favor the second hypothesis stated in the introduction, that of a primary effect common to all organs with a superimposed hormonal effect. It must be considered, however, that the precise degree of proportionate response to hormonal influence has not been experimentally established in this organism and that a considerable margin of error should be allowed for in the percentages. In view of these points, it is felt that a conservative conclusion would limit inference of the presence of a primary effect in addition to the hormonal effect to the salivary gland, where an unmistakable effect exists, while suggesting or at least not precluding the possibility of such an effect in other organs, where it could be masked by the hormonal effect. Significant in this respect, as well as for its own sake, would be the use of the DNA quantitative index to determine the response to various conditions of hormonal influence.

This state of affairs, unresolved as it is, is not the impasse it seems. Let us ask the question: what characteristic is common to all the organs in which a primary effect is clearly indicated, that is, to the salivary gland and possibly the brain from the present work and to the gonads and imaginal discs, as well as the salivary gland, from previous work? The answer is that they all have the need to synthesize large amounts of DNA, the salivary gland in its functional aspect, the gonads in premeiosis, and the other organs in multiple mitoses. It may then be deduced that the primary effect common to these organs is one that results in some impediment to the synthesis of DNA. In itself, this is pure deduction, but it is not unsupported by experimental evidence. It will be recalled that the reduction of DNA in salivary gland nuclei seems disproportionately large in comparison with the reduction of total cell volume. This, however, would be the logical consequence of a primary effect that is concerned with the synthesis of DNA itself. Experiments are underway at present employing aminopterin with the purpose, in particular, of attempting to produce a phenocopy of the *l(2)gl* mutant and, in general, of investigating differences in DNA synthesis in the various cell types of *Drosophila melanogaster*.

If this, indeed, is the identity of the primary effect, some additional features can be filled in. As it affects diploid as well as polytene nuclei, it must be concerned with the general synthesis of DNA rather than with some aspect associated only with polyteny. In the salivary gland, as DNA reduction is not followed by a corresponding reduction of cytoplasm it must be concerned here with the synthesis of DNA in its metabolic rather than genetic aspect, the latter of which should be proportional to cell size.

As the prothoracic hormone has been linked with the cytochrome system, the deficiency of hormone in this mutant could well set a limit to the oxidative capacity of the various organs and so account for the lowered oxygen consumption found. Experimental evidence for this conception is the fact that the difference between DNA reduction and cytoplasmic reduction in the salivary gland is most marked at the 120 hour stage, at which time the lethal ring gland has had an opportunity to reach its maximum capacity and furnish a maximum of hormone for cell growth.

If such, indeed, are the natures of the primary and hormonal effects, they are related only in the sense that each is involved in the total economy of the cell.

If the primary effect is concerned with the synthesis of DNA, it will be present in all organs, but in those organs in which the synthesis of DNA is relatively low, the primary effect might not have enough weight to differentiate itself from the overall hormonal effect. This is apparently the situation in the alimentary system, fat body, muscle, and epidermis. The fact that tissues such as the corpus allatum and the somatic cells of the gonads show normal metamorphosis on transplantation to a normal host could be explained by the receipt of some essential substance from the host, a possibility which is conceded though not favored by HADORN (1951), or by a deficiency in nucleic acid synthesis that might not be severe enough to prevent normal growth or might even be further discounted in pupal than in larval metabolism.

Ubiquity of effect would be a natural consequence of a fundamental interference in the growth process such as seems to be present in this lethal mutant. The fact that it does exist provides the necessary premise to the hypothesis that there is one primary effect from which the entire pattern of damage is derived. Proof of this hypothesis involves determination of the nature of the primary effect. Previous work as to the existence of a primary effect more basic than the hormonal effect in such as salivary gland, imaginal discs, and gonads, through transplantation of these organs into a normal host with little or no effect, and also present work concerning the effect on the brain and the differential effect on the salivary gland in comparison with other organs examined suggest the observation that the organs primarily and most severely affected are those that need to synthesize DNA most heavily. In view of this it seems a reasonable probability that the primary effect is concerned in a direct rather than generalized manner with the synthesis of DNA. This opinion finds some experimental confirmation in the selective effect on the salivary gland nucleus with regard to the cytoplasm. While the evidence does not conclusively establish, it does suggest the reasonable probability that in the *l(2)gl* mutant there is a primary effect common to all tissues concerned with the synthesis of DNA, and a secondary, superimposed hormonal effect arising from the operation of the primary effect in the ring gland. Thus, the syndrome of retardation in this mutant would be classified as "spurious" or "cell-reactive" pleiotropism resulting from unitary gene effect.

SUMMARY

1. Cytophotometry of Feulgen stained preparations, employing the two wave length method, has been used to analyze the retardation in development of the lethal mutant *l(2)gl* in *Drosophila melanogaster*, in comparison with its viable heterozygote and with the normal Stephenville wild type larva.

2. Test measurements on rat liver nuclei establish that the accuracy of the method as practised in this laboratory is represented by a coefficient of variation of from eight to ten percent.

3. Measurements on brain nuclei establish a diploid value in accordance with which values for other types of nuclei are grouped into polytene classes.

4. Variation in DNA content of Drosophila nuclei, including experimental error, is represented by an average coefficient of variation of about 15 percent.

5. On the basis of measurements on nuclei of ring gland and salivary gland, no significant difference is found between the viable heterozygote of the lethal strain and the Stephenville wild type larva.

6. Sex differences cannot be distinguished, and individual differences are slight. The larvae show great uniformity, both in speed of development and at maturity.

7. By means of DNA as a quantitative index, the course of development of both normal and lethal larvae is charted.

8. The chief conclusions from application of the DNA quantitative index to the *l(2)gl* mutant are as follows: the lethal effect is ubiquitous, affecting all organs; the ring gland nuclei are about 60 percent of normal at the 96-hour stage; retardation in corpus allatum, alimentary system, fat body, muscle, and epidermis nuclei is roughly correlated with that in the ring gland; a marked differential effect is evident in the salivary gland, the nuclei of which, in terms of DNA, are only about 20 percent of normal; and DNA measurements, combined with qualitative observation show that in the salivary gland the DNA is disproportionately reduced in comparison with total cell volume, this effect being particularly marked at the 120 hour stage.

9. In combination with previous work as to the influence of the ring gland on pupation, the evidence, it is felt, incriminates beyond reasonable doubt the ring gland as the agent that retards larval development of the lethal mutant through superimposed hormonal influence probably related to the oxidative metabolism of the cell.

10. The evidence suggests the reasonable probability that the primary effect, from which the hormonal effect arises in the ring gland, is common to all tissues and concerned with the synthesis of DNA. Under this interpretation, the *l(2)gl* mutant would be an example of "spurious" or "cell-reactive" pleiotropism originating in unitary gene effect.

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