

DEVELOPMENTAL STUDIES ON LETHALS
IN DROSOPHILA.

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PART 1

MALIGNANT AND RELATED PATTERNS
IN DROSOPHILA.

EDWIN STON

EXCURSUS STRONG

PART 1

TABLE OF CONTENTS

Introduction	1
Materials and methods	13
Stocks	13
Egg collection	15
Histological technique	16
Blood-forming organs in <u>Drosophila</u>	17
<u>Lethal malignant</u>	22
Histology and behaviour of <u>lethal malignant</u> lymph glands	22
Primary aggression	25
Metastasis	26
Secondary aggression	28
Other abnormalities characteristic of the malignant phenotype	30
Wild-type testes	31
Anti-neoplastic activities	33
Type 1	37
Type 2	38
Type 3	39
Test of malignancy diagnosis	41
Formation of the black bodies	43
Behaviour of the imaginal discs in larvae in which the tumours were inhibited early	46
Contributions of the lymph gland cells to normal development	48
Imaginal primordia	49
Imaginal disc mesoderm	53
The ventral hypodermal organs and the circulatory system	56
Blood cells	59
<u>Lethal no-differentiation</u>	61
Imaginal discs	63
<u>Lethal benign</u>	69
Blood-forming organs	71
The final stages in the development of the <u>wild-type</u> lymph glands	74
Discussion	77
Summary	100

INTRODUCTION

Genetics and Cancer Research

Despite the growing attention given to malignant diseases, the mechanism of their causation remains obscure. The contributions of pathology have been valuable mainly in clinical diagnosis and the findings obtained from purely medical data have little bearing on the biological nature of tumour growth. Recently geneticists have begun to give serious attention to neoplastic proliferations, in the attempt to attribute them to genic causation. Work on tumours is being carried out on almost every material suitable for genetic analysis.

Little (1909) developed the well known high tumour strain dba in mice. Since then several strains with high and low breast tumour incidence have been established. The use of inbred lines has not been restricted to the study of mammary cancer but expanded until to-day there are around seventy or more isogenic strains of mice used in work on the different neoplasms. The different types of tumour growths being studied in mice are breast tumours, pulmonary tumours and subcutaneous tumours. However, Strong (1945) reported his development of a strain of mice with a high incidence of gastric carcinoma through a genetic procedure aided by routine injection of methylcholanthrene. This is of some importance, since previously only strains of

mice with a high incidence of adenoma of the glandular stomach, which is benign, had been available.

As yet there is no conclusive evidence that any tumour of the mouse is inherited by only a single gene. Instead, multiple-factor inheritance appears to be involved in every case. Evidence against single factor inheritance is derived from the variation in degree of susceptibility as expressed by the variation in tumour incidence of the different strains, together with the more conclusive data obtained from hybridisation studies. Reciprocal hybridisation between several high and low mammary tumour strains in mice revealed that the tumour incidence in the females resembles that of the strain of the mother. Since the genetic constitution of reciprocal groups of F1 females, resulting from crossing two isogenic strains was the same, it was obvious that this effect was an extra-chromosomal factor which the female was able to transmit to her offspring. Bittner (1936, 1937a, b and c), by his foster nursing technique, identified this non-genic factor and demonstrated it in the milk of the high-tumour strain mothers. This revealed the important role of the milk agent in determining the susceptibility to mammary tumours in mice.

Drosophila tumours

A number of genetically determined tumours in

Drosophila larvae and adults have been described by several workers (reviews in Burdette, 1950a; Sharrer and Lochhead, 1950). Some of them have appeared in connection with lethals (Bridge, 1916; Rapoport, 1938; Stark, 1918; Russell, 1940); while others are benign tumours causing the larvae no harm (Brncic, 1949; Ghelelovitch, 1950; Russell, 1942; Stark, 1919a; Wilson, 1924).

Most of the tumours which have been described by the different workers have shown a histological identity suggesting a common origin and a common mode of formation. They appear invariably as freely moving black bodies occupying the caudal portion of the haemocoel of the larvae. The black pigment is probably melanin (Hartung and Tillinghast, 1949). Their sudden appearance occurs about the time of puparium-formation; that is to say, they appear at the closing hours of the late larval instar and persist in the adult in the same larval foci as empty black capsules.

Attention has recently been paid to the analysis of the inheritance of tumours in the various tumour strains. Russell (1942), by a technique of substituting marked chromosomes carrying crossover reducers, was able to demonstrate that the tumour factor in the sr es ro ca/tu 36a strain is located in the second chromosome and that modifying factors are present in

the first and third chromosome. Ghelelovitch (1950) indicated that the fat body tumour depends on a genetic factor located on the left arm of chromosome 2, and that modifiers are present on the third and possibly the first chromosome. The tumour phenotype of the stock $tu(2)^{49K}/ma\ 49d$ is controlled by a recessive factor on the second chromosome, and modifiers in the first and third (Ofstedal, 1953). Hartung (1950) has roughly determined the locus of the main factor and of the modifiers in the $bw\ tu$ tumour strain.

While these results suggest a multiple-factor inheritance of the tumours involved, Bridges (1916) showed that the tumour $l(1)7$ was inherited as a sex-linked recessive lethal mutation.

Theories of malignant growth

Speculations on the nature and causation of malignant new growths fall into three groups:

- 1) The genetical hypothesis originating with Boveri, and modified by Bauer;
- 2) The virus hypothesis particularly associated with the names of Borrel, Rous and Gye;
- 3) The special form of the chronic irritation theory of Virchow which has emerged on the basis of the work of Yamagiwa (1905) on the experimental production of tar cancer.

1) The somatic mutation theory of cancer was put forward as a result of Boveri's well known studies on

the development of disperme-fertilised echinoderm eggs. He showed that the haploid number of chromosomes or any multiple of it was compatible with normal development, but that pathological conditions arise in the disperme eggs, which may be attributed to chromosome abnormalities, one or more of the four primary blastomeres having received an incomplete set of chromosomes from the multipolar segmentation division. He suggested that the abnormal distribution of the chromosomal complex might cause a loss of the factors which normally inhibit the rate of cell growth. However, as pointed out by Morgan and Bridges (1919), such chromosomal aberrations are not necessarily associated with cancer growth. Subsequent efforts to support the theory have completely failed. Koller (1947) who, investigating the cytology of various human tumours, found that the number of chromosomes had a range of 12-48, with a frequency peak at about thirty and another, but lower, peak at forty-five chromosomes. But it is clear that mere abnormality of chromosome number cannot be held responsible for the causation of tumour growth. This was shown, for example, by the work of Timonen and Therman (1950) and Therman and Timonen (1951) whose cytological studies of human normal somatic tissues, (proliferative stage of the adult uterine epithelium and embryonic tissues), showed that the chromosome number varied considerably and that the number 48

generally held to be constant, is not the most common outside the germ line.

It has therefore been found necessary to shift the assumed damage to individual genes, an assumption which geneticists have not yet been able to prove.

2) The virus hypothesis. The modern discussion of this hypothesis revolves around the study of the filterable tumours of the domestic fowl, first discovered by Rous (1911). He described four strains of sarcomata, all transmissible by cell-free filtrates. Subsequent workers, according to a review by Murray (1933), have brought the number up to thirty. Their most remarkable feature is the biological and histological constancy of the growths propagated by cell-free filtrates, and the multiplication of the active constituent of the filtrates during growth in the susceptible animal. The latter character points to a living self-reproducing agent, which may be defined as a virus.

3) The experimental confirmation of Virchow's chronic irritation theory has established its validity as a concise account of the emergence of cancer after prolonged localised irritation of the tissues by a variety of agents. The constancy of the results of the experimental production of cancer, and the frequency and restriction of certain cancers to those following definite occupations involving exposure to the same substances, fully justifies naming these

causative factors as carcinogenic agents. Their chemical properties give no indication of how the autonomous, uncontrolled type of cell proliferation is induced. Indeed, they are so varied that it is difficult to see how they can give any definite indication of the nature of the cellular mechanism involved.

A note on the diagnosis of malignancy

From the systematic viewpoint, tumours fall into two big divisions; the one innocent, simple or benign; the other malignant. This is true both for epithelial and connective tissue tumours. In the former carcinoma, and in the latter sarcoma is the malignant representative. Though malignant and benign tumours belong to entirely different groups of neoplasms, yet papillomata are known which change their benign feature to become malignant, i.e. colon papilloma occasionally changes to a carcinoma. An innocent tumour grows by expansion and is usually separated from the surrounding tissues by a capsule, causing eventually no harm to its carrier. Malignant proliferations, on the other hand, are characterised naturally by displaying aggression on primary tissues, and later by metastasis on secondary tissues. There are several other major points of difference, pointed out in detail in any book of medical pathology.

Malignant tumours offer no direct diagnostic test

similar to that of tuberculin in tuberculosis, in which a reaction peculiar to tuberculous animals becomes apparent. The common test of malignancy depends on the technique of biopsy, and involves the removal of a piece of the suspected tissue for microscopic investigations. Most of the neoplasms exhibit histological features which permit a clear-cut diagnosis, the difficulty of discriminating between hyperplastic and neoplastic growths being limited to rare special cases.

Starting from a quite different point of view, far from those of classical histology, Caspersson and Santesson (1942) succeeded in finding an original method of differentiating between malignant and normal cells. Using monochromatic ultraviolet light, they were able to show that in the malignant tumour mass two different kinds of nuclei can be found (the proliferating and the pre-necrotic, labelled as A and B respectively). The former type appears rich in nucleic acids, which seem to be much less in the second, or B type, in which the remnants of nucleic acids are confined to the big nucleolous, only scanty traces being present in the remaining parts of the cells. Both types are connected by a continuous chain of intermediate stages. The same results were reached by Koller (1947) using the simple technique of the smearing of biopsy material after treatment with carmine or Schiff-Feulgen reagent.

Barigozzi and Dellepiane (1951) took special care

to differentiate between malignant tumours and hyperplasia of the endometrium. A sharp difference was found, because in most instances no similarities existed between malignant and hyperplastic cells. On the other hand, they found no difference between normal and hyperplastic epithelial nuclei. According to the same authors, the structure of normal nuclei, for epithelial cells at least, seems to be very uniform. The presence of one or two chromatic masses is assumed to be taken as a good index of normality. In the proliferating A nuclei of malignant cells, there is generally a very high and not precisely determinable number of these masses. The non-proliferating nuclei (type B cells), having a tendency to necrosis, are practically devoid of these assumed chromocenters and the chromatin threads can be seen only with difficulty. These cytological criteria of malignant cells have been obtained from studies on cancers of the uterus. On the other hand, Koller's findings did not cover all types of neoplasms. However, Barigozzi and Casabona (1948) and Casabona (1949) made a series of comparative investigations on different neoplasms of the rat out of which they assumed that the cytological pattern at the nuclear level does not differ in the different animal species. If these cytological findings obtained from some neoplasms be found to constitute a common feature of all malignant tissues, they could provide a

diagnostic test, and might overcome the difficulty where the histological criteria alone are not sufficient to distinguish between some neoplasms. For example, difficulties are usually encountered in the classification of adenoma versus adenoma malignum, fibroma versus fibrosarcoma, leiomyoma versus leiomyosarcoma, etc.

Cancer and physiological genetics

Although the occurrence of hereditary factors affecting susceptibility to tumours is usually assumed, neither Drosophila nor any other material used in tumour work has exhibited them clearly in the form of classical gene segregation. This is probably due to factorial complexes being involved, and to the stimulation of malignant proliferations by virus and carcinogenic agents, which have attracted so much interest as to turn our attention from possible gene interference.

Physiological genetics deals with genes and their processes of action. Genes act as phenotypic determinants by controlling sequences of biochemical reactions by which the complicated processes of development are initiated and organised. This naturally does not apply only to processes of increase; the whole general physiology of the individual is merely the expression of the activities and interaction of its genes. Any important aberration, therefore, in a genic complex reflects itself as a lethal factor exhibiting a pattern

of damage. Such lethal factors are known and a few have been studied in Drosophila. Of the factors affecting post-embryonic stages, lethal giant larva (lgl) was studied by Hadorn (1937a and b, 1938), Hadorn and Gloor (1942, 1943) and Gloor (1943); lethal-cryptocephala was studied by Hadorn and Gloor (1943); lethal-translucida was investigated from different aspects by Hadorn (1948, 1949), Gloor (1943), Chen (1951) and Sobels and Nijenhuis 1953; while lethal-meander was roughly described by Hadorn (1943) and was later discussed in detail by Schmidt (1949). Although the technique of transplantation was used, the causes of lethality have usually been difficult to determine, although in some cases an interference of the lethal factor in the general endocrinology of the eventually lethal individuals was suggested.

Lethal factors producing malignant growth have not been described in Drosophila. However, their occurrence amongst the steadily increasing number of lethal factors should not be unexpected, as neoplasia are not supposed to be restricted to mammals. If we bear in mind that cancer should preserve its characteristics even in Drosophila, we may expect that we shall find it necessary to deviate from the conventional conceptions of Drosophila tumours and develop a more mature idea of what neoplastic growths in Drosophila should be like. In view of the

simplicity of Drosophila, genotypically as well as structurally, one might hope that any such lethal pattern could be easily studied and that it might throw light on fundamental problems, such as the biological nature of malignant growths.

Though Drosophila is extensively used in tumour work, it has not been thoroughly investigated whether the pigmented bodies identified as tumours are really so. These melanotic tumours are generally regarded as cell proliferations of unknown origin. This has not been confirmed by histo-pathological procedures. So far, indeed, the histogenesis of these tumours remains obscure. In spite of our ignorance of their true nature, they provide the only material usually used in Drosophila tumour research. This thesis deals mainly with tumours in Drosophila. For the first time a malignant tumour is described; it appeared in connection with a sex-linked lethal. Lethal larvae of another lethal stock are also studied from the tumour point of view, and compared with the malignant larvae, since they show the formation of pigmented bodies at the end of the third instar. A third tumour-free lethal stock is also considered because of the involvement of the tumour mother organ in its lethal phenotype.

MATERIALS AND METHODS

The work to be described is based on the investigation of three sex-linked lethal mutations in Drosophila melanogaster. These are (1) lethal-malignant (symbol l-m); (2) lethal-no-differentiation (symbol l-n-d); (3) lethal-benign (symbol l-b). For none of these lethal factors has its exact locus on the X-chromosome been worked out, nor has there been any attempt to determine cytologically whether they are point mutations or chromosome aberrations. All three mutations were induced at the Institute of Animal Genetics, Edinburgh, probably by mustard gas, and were selected for further investigation after a preliminary examination of the numerous sex-linked lethals which are discarded by the workers concerned with problems of induced mutation.

Larvae hemizygous for lethal-malignant continue to survive as larvae for not more than four days after the normal time of puparium-formation. Some may die as larvae after this period, while others form puparia and die either as early pre-pupae or early pupae. The larvae of lethal-no-differentiation behave in a similar manner, except that puparium-formation is not delayed for more than two days. The larvae of lethal-benign consistently die by the time of normal puparium formation. Some of them, however, may form pseudo-puparia, after which development proceeds no further. True puparium-formation was

not observed.

The lethal factors were kept balanced against the Muller-5 chromosome which functions as cross-over inhibitor since it carries an inversion. It also contains Bar (B), a semi-dominant gene, the heterozygous females being easily distinguishable from the homozygous ones. Further, the chromosome contains the gene apricot (w^a). Stocks are carried on by selecting in each generation heterozygous Red Bar females which must contain the lethal in their other X-chromosome. The cross is illustrated genotypically as follows:-

$$\begin{array}{c} \frac{M-5}{1} \times \frac{M-5}{7} \\ \\ \frac{M-5}{M-5} \quad \frac{M-5}{7} \quad \frac{M-5}{1} \quad \frac{1}{7} \\ \text{die out} \end{array}$$

As shown above, the lethal phenotype is exhibited by half the males. The sex of the larvae can be distinguished by the size of the gonads, the testes being strikingly bigger than the ovaries at a very early larval stage. The presence of the apricot gene in the Muller-5 chromosome allows one to distinguish the lethal-bearing male larvae from those carrying the harmless Muller-5 chromosome. Apricot is an allele of white and it is known from the work of Beadle (1947a and b) and others that larvae homozygous or hemizygous for white have colourless Malpighian tubules, whereas those of wild-type larvae are bright yellow. A similar effect on the colour of the

Malpighian tubules is exhibited by a number of other eye-colour mutants such as ruby, carmine, garnet, light, peach, claret, etc. The lethal-bearing males which will exhibit the lethal phenotype are those larvae with large gonads and bright yellow Malpighian tubules.

Egg Collection

Well-fed flies not less than five days old were transferred to milk bottles which were then inverted over watch glasses containing a 3% agar solution to which 1% acetic alcohol had been added. The surface was shaved off to provide a rough surface for egg deposition. No yeast was placed on the agar lids. Hatched embryos were collected over one half hour and the age of the 1 larvae could thus be determined within 15 minutes. Culture dishes were prepared of 50 mm. petri-dishes lined with filter paper and containing ordinary *Drosophila* food. The filter paper was soaked regularly with a fine emulsion of yeast in water to keep the food as fresh as possible.

The life cycle of *Drosophila melanogaster* from egg hatching to the emergence of the imago is about 192 hours at 25^oC. The larvae pass through three instars and two larval moults. The first moult occurs at about 25 hours, the second at 48 hours after hatching, and puparium-formation, which marks the end of the third instar, at about 96 hours. For approximately 12 hours after puparium-formation the enclosed individual

enclosed individual within the puparium is termed a prepupa. The pupal stage covers a long period which starts from the end of the prepupal stage and continues till the imago is due to hatch. For accurate timing within the third instar, the actual occurrence of the second moult was observed and timings made from that.

Histological Technique

Fixation of specimens for sectioning was carried out in hot fixatives. Boiling water gave as satisfactory results as did hot Bouin at 80°C. However, the latter gives better results in that it softens the cuticle and other hard structures which resist infiltration by wax. To overcome the impermeability of the cuticle to Bouin, specimens are pricked in the posterior part of the abdomen by a fine tungsten needle, and are kept in the fixative for at least 24 hours. After washing out the excess of Bouin by several changes of 70% alcohol, dehydration proceeds through successive grades of ethyl alcohol. A modified Peterfi's celloidin-paraffin technique was found useful, as it avoids the distortion or rupture usually encountered with ordinary paraffin embedding. The larvae were transferred from absolute alcohol to .5% celloidin in methyl benzoate for 24 hours. This was followed by two transfers through 1% and 2% solutions successively, each for at least 24 hours. Clearing was done in benzene and embedding in hard wax containing ceresin and stearic acid. Sections ranged from 2-5 microns and were

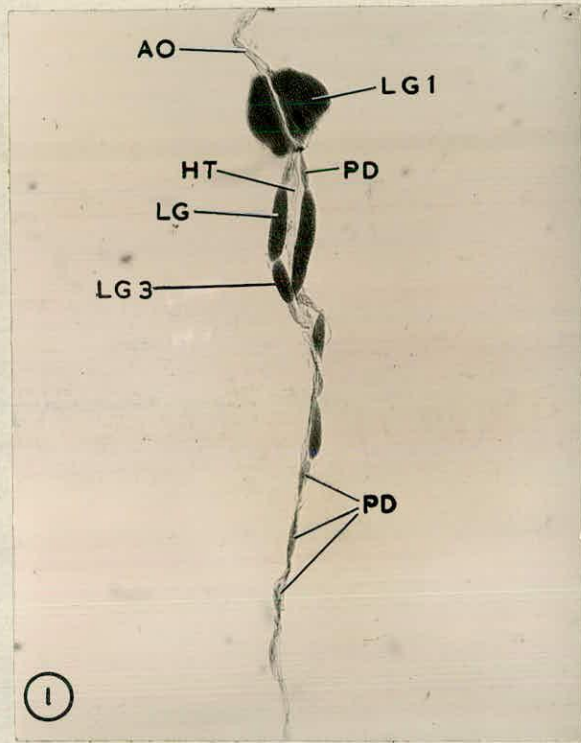
stained in Delafield's haematoxylin with eosin as a counterstain. The ethyl-butyl phenol technique recommended for insect eggs (Darlington and La Cour, 1942, after Smith 1940) was occasionally used for dehydration purposes and provided good results.

Blood-forming Organs in Drosophila

In all three lethals the structures most profoundly affected are the so-called blood-forming organs. It is believed that the changes produced in them are the primary ones brought about by the lethal factors and it is therefore necessary to describe them in some detail. Before this is done it is necessary to give an account of the structure of the organs in the normal wild-type Drosophila.

The dorsal blood vessel and associated structures in Drosophila have been described by several workers (Poulson, 1945; Robertson, 1936; Stark & Marshall, 1928). The accounts available in the literature are, however, neither precise nor complete and the following description is based on new histological material. Preparation of the dorsal circulatory apparatus is shown in Fig. 1. It does not seem to differ essentially from that of other dipteran larvae as described by Weissmann, Wandalleck, Lowne, Giacomini and others. There is a simple tubular heart which is continued anteriorly as a dorsal aorta, which penetrates

Fig. 1. A whole mount of the dorsal blood vessel and associated glands obtained from 75 hour old larva. AO - aorta; LGI - first pair of lymph glands; PD - pericardial cells; HT - pulsatory heart; LGII - third pair of lymph glands. Notice that the second and third lymph glands of the right side are fused while those of the left side are separate. X 70.



the ring gland on its ventral side and opens forward into the body cavity. A short distance posterior to the cerebral hemispheres and directly above the proventriculus the heart is flanked by two types of paired organs which are commonly known as the blood-forming organs or lymph glands (Stark & Marshall) and the pericardial cells.

Studies of a number of whole mounts revealed the basic morphological pattern, which is of normal occurrence and is illustrated in the Fig. . The anterior pair of lymph glands is the largest and appears as a triangular trilobated thin structure. The second and third pairs are tubular, much smaller in size and connected with each other by fine ligaments. Posterior to them are a fourth and a fifth pair, which are slightly smaller in size than the third. There is a pair of pericardial cells located between the first and second pair and in direct connection with their ends. A second and third pair of pericardial cells connect the third and fourth, and fourth and fifth, pairs of glands respectively. As the abdomen is composed of 8 segments it becomes apparent that the lymph glands are not segmental. Next to the last pair of glands, the heart is bordered by a double row of pericardial cells which show a constant number of ten pairs. The cells of the anterior 5 pairs, as well as those of the other three pairs in between the glands, are fusiform with gradually

tapering ends. The cells of the posterior five pairs are very close to each other, and are rounded in shape, with the exception of the first which is transitional.

Variations however from this general morphology are not infrequent. The first pair of pericardial cells may be absent, with the result that the first pair of glands is in direct connection with the second. In such cases however, the second and third may either fuse completely to form an unsegmented pair, or fuse incompletely leaving a constricted suture. Specimens without the third pair of pericardial cells showed the fourth pair of lymph glands followed by a fifth and a sixth pair.

Some preparations showed the fourth and fifth pairs of glands fused together. The maximum number of blood-forming organs found (in 25 cases investigated) did not exceed six pairs.

This description was based on material slightly older than 82 hours after hatching.

The internal histology of the glands is important for our investigation. In late third instar larvae the histological structure is already somewhat degenerate and it was impossible to distinguish clearly between the different types of cells, although one can recognise what Stark (1928) referred to as "large and small blood cells". More structure can be seen in younger glands (about 75 hours after

hatching). The very small size of most of the cells and their vigorous affinity for basic dyes makes them a difficult histological material. With care, however, these difficulties can be largely overcome. It was found that all five pairs of lymph glands have similar structure and contain three main types of cells which may be described as follows:-

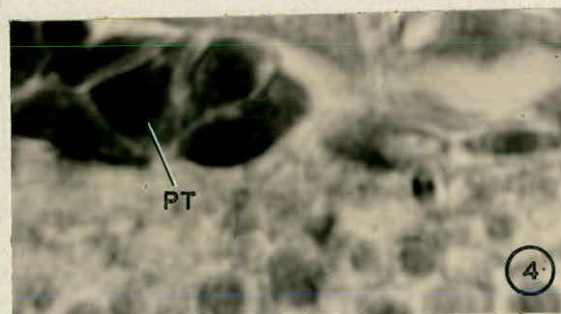
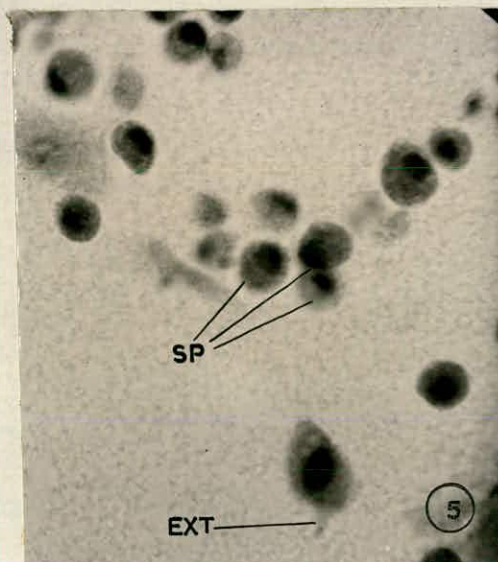
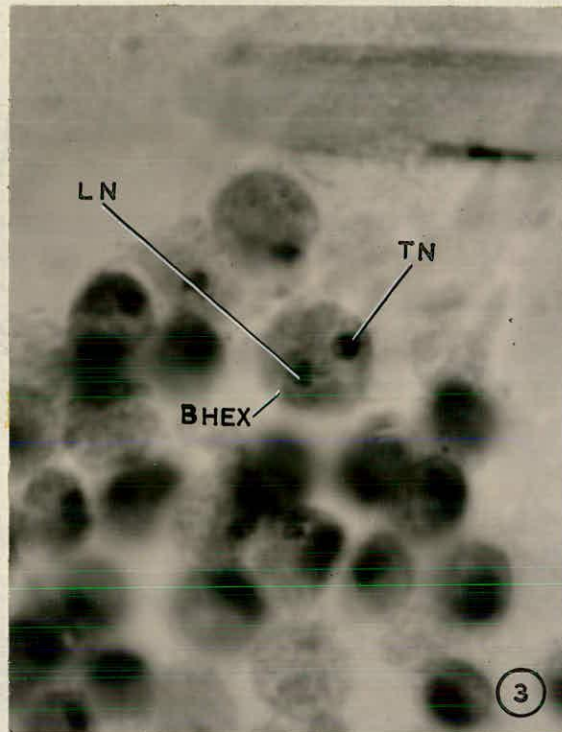
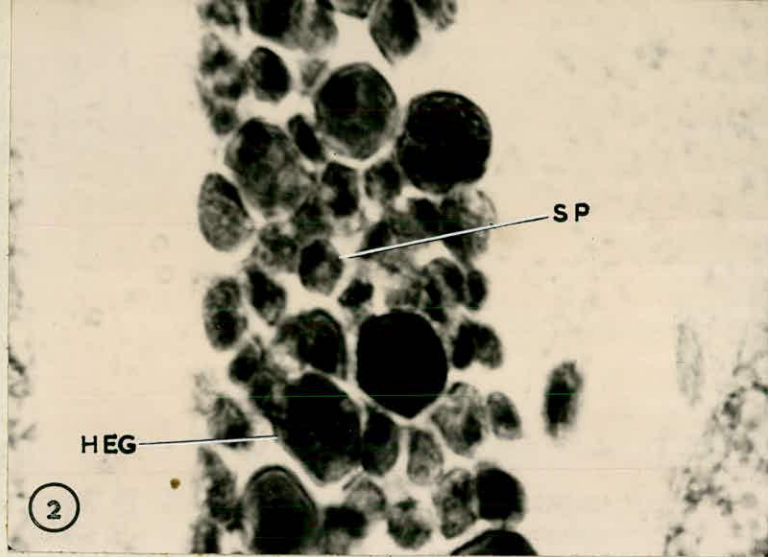
(1) "Hexagons" (Fig. 2). These are large cells roughly globular in shape. One side of the cell, the base, is rounded, the other is rather rounded in the shape of a truncated pyramid erected on a hexagonal base. This hexagonal shape is often strikingly obvious when the cells are seen in sections and it is in reference to it that they have been named hexagons. The cells sometimes contain one but sometimes two nuclei. In the mono-nucleated hexagons the nucleus lies near the truncated tip of the hexagonal pyramid. These nuclei are large and stain deeply with basic dyes. They are referred to as "upper nuclei". In the bi-nucleated cells (Fig. 3) one nucleus always occupies this upper position. The other lies towards the side of the cell in the region of the rounded base, its nuclear membrane is roughly rectangular in outline and the nucleus is slightly smaller than the other one. In both types of cells the cytoplasm is a little less basophilic than the nuclei. Both types are relatively rare and occur scattered among the remaining lymph gland cells.

Fig. 2. A highly magnified portion of control 75 hour old lymph gland. SP - spheroids; HEX - hexagons. X 1500.

Fig. 3. A binucleated hexagon (BHEX) showing top nucleus (TN). LN - lateral nucleus. X 2200.

Fig. 4. A platlet cell (PT). Notice triangular outline. X 2000.

Fig. 5. A semi-squashed preparation of control blood-forming organs showing different views of spheroids. SP - spheroids showing globular outline and EXT - basal extension. X 1000.



(2) "Platelets" (Fig. 4). These cells are smaller, more or less triangular in shape, with the wider side of the triangle slightly curved, while the other two sides are somewhat irregular in outline. They contain only one nucleus. They are relatively rare cells occurring mainly at the posterior end of the lymph glands. They are more basophilic than the hexagons.

(3) "Spheroids" (Fig. 5 from a stained, semi-squashed whole mount). These are small, more or less globular cells, usually with a short cytoplasmic extension, pushed out from the basal part of the cell body. There is a large nucleus with a rounded membrane lying at the opposite side of the cell. None of the cells seems to contain more than one nucleus. They are the most frequent type of cells in a lymph gland tissue. They stain less deeply than the platelets.

The lymph glands hang freely in the haemocoel and are encapsulated by a very thin epithelium, probably of squamous cells. The lymph gland cells which have just been described are embedded in a uniform stroma. The nature of this is somewhat obscure. In the glands as they normally exist, the dense mass of cells makes the stroma almost invisible. It can, however, be seen when the cells are partially separated when a gland is pressed under a cover slip. In view of the open type circulation the stroma is, of course, not vascularised. It is, however, provided with tracheal supplies.

Lethal-malignant

In lethal-malignant the first and second instar larvae seem to be morphologically and histologically perfectly normal. No obvious external lethal effect can be seen even in the third instar larvae. However, the puparium-formation is delayed from two to four days and in a few cases it is preceded by the appearance of black bodies within the body of the larva. The lethal individuals die either as prepupae or early pupae.

Histological examination shows that before this there have been considerable internal abnormalities which can be seen first in the lymph glands.

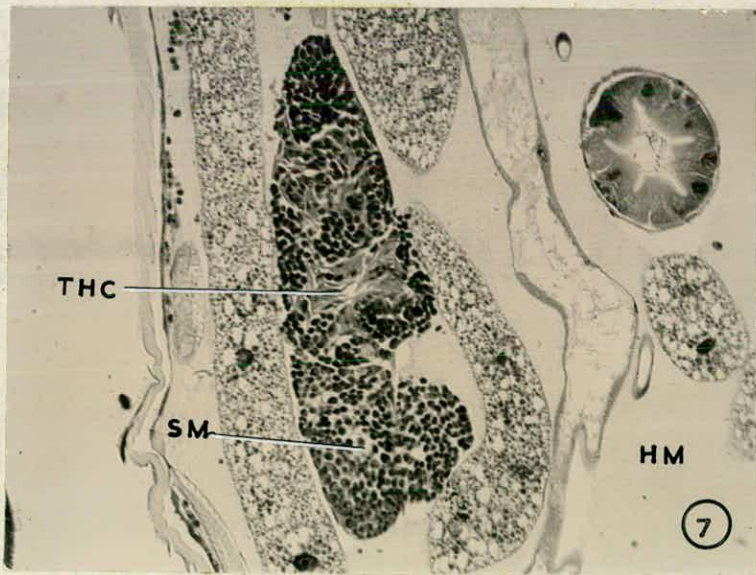
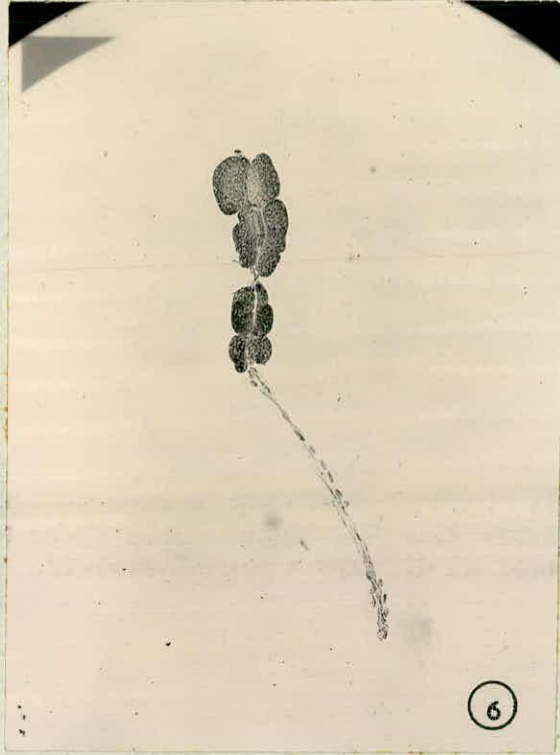
(1) Histology and behaviour of lethal-malignant lymph glands.

In the early part of the third instar the histology of the lymph glands is normal. Deviation from normality occurs in the later part of the third instar when the lymph glands increase enormously in size and within a period of 24 hours become several times as large as normal (Fig. 6 compared with Fig. 1). In sections it can be seen that this increase in size is connected with active multiplication of both spheroids and hexagons. The latter, which in normal glands are represented only by a few scattered cells, in the lethal comprise a good portion of the lymph gland tissue.

In the lymph glands of 5-day old larvae (i.e. about one day after the normal time of puparium-formation) a new type of cell makes its appearance in the gland.

Fig. 6. A whole mount of 5 day old lethal malignant lymph glands.
Notice enlarged size and compare with those of Fig. 1.
Both are taken at different magnifications. X 55.

Fig. 7. A section in a $5\frac{1}{2}$ day old lethal blood-forming organ.
THC - thread cell clusters. X 500.

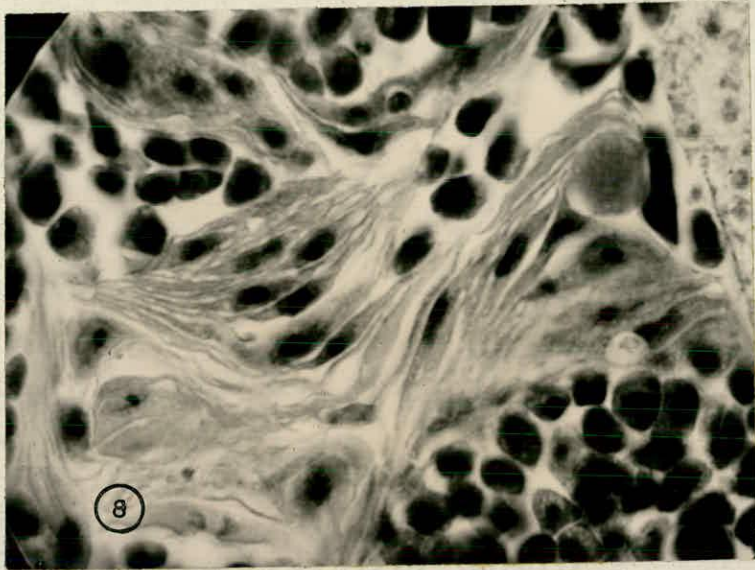


They appear first near the centre (Fig. 7) and later approach the periphery. Fig. 8 shows them under higher power and reveals clusters of elongated cells. Each of these cells at this stage has a wide, oblong central portion containing a nucleus which is of considerable size and is highly basophilic. The ends of the cells are prolonged into long twisted arms which taper gradually and appear often to be attached to one another at their extremities. These cells are referred to as "thread cells". After a few clusters of these cells have been formed they seem forcibly to penetrate the outer epithelium of the gland and thus to cause a general release of the lymph gland cells. The thread cells themselves also pass into the body cavity where they may be seen either fully extended or with one of their long arms bent back over the main cell body (Fig. 9). They remain intact in the body cavity only for a short time, after which they degenerate and are finally represented only by non-nucleated thread-like structures (Fig. 33).

In rather earlier stages, i.e. in larvae whose age corresponds to a late third instar, appearances can be seen, which probably represent the cell divisions which give rise to the appearance of the thread cells. These divisions occur in the binucleated hexagons and separate the lateral and upper nuclei. The plane of division is curved and the two daughter cells are

Fig. 8. The central portion of Fig. 7 under a high magnification.
Notice the nature of the thread cells. X 1200.

Fig. 9. Thread cells as they appear in the haemocoel. Some
of them show their ends bent on the main cell body. X 900.



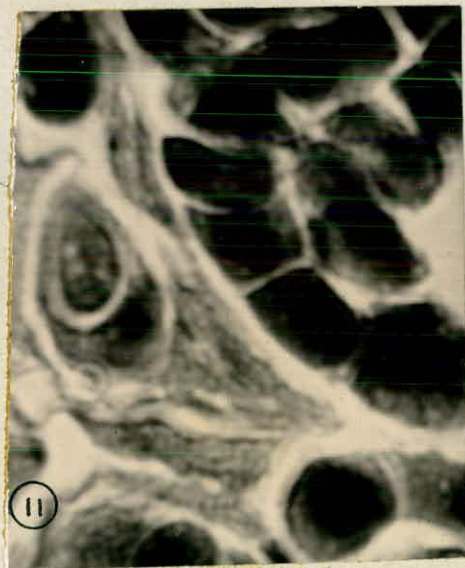
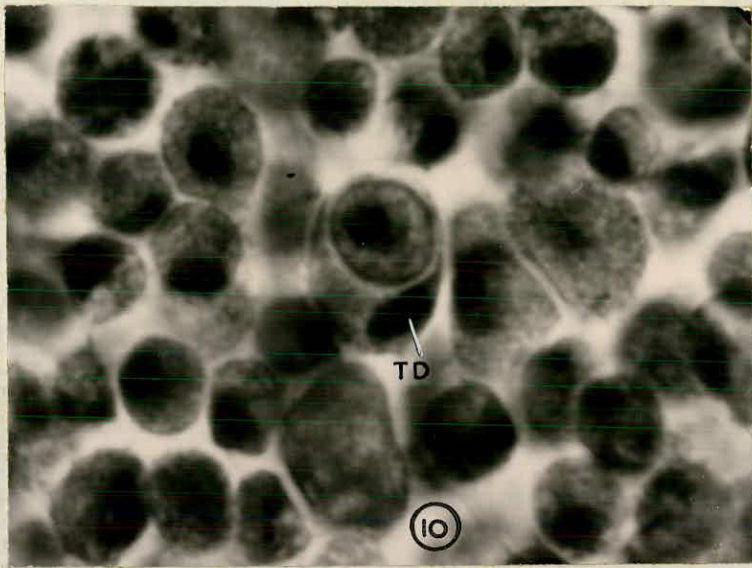
different in size and shape. In Fig. 10 a rounded cell containing the upper nucleus is partly enveloped by a crescent-shaped cell containing the lateral nucleus. It seems probable that these ~~two~~ cells have originated by the division of one binucleated hexagon and that the crescent-shaped cell is a precursor of a thread cell. Intermediate stages between such relatively compact crescent-shaped cells and the fully extended thread cells can be found. Fig. 11 shows another rather similar type of division, but in this case the crescent-shaped cell envelopes the round one even more completely. This type of cell division is, of course, a peculiar one. Since both nuclei are present before it occurs, it does not involve mitosis. Abnormal types of cell division or cell cleavage are, however, not uncommon in relatively highly differentiated tissues, such as those of the lymph glands. At this stage both hexagons and spheroids show big hyperchromatic nuclei and become irregular in shape and show signs of early loss of differentiation.

Release of the lymph gland cells.

The development of the thread cells leads to the rupture of the outer epithelium of the gland, often in several different places. The stroma, particularly of the posterior half of each gland, becomes loose in texture and is visible often for the first time. Simultaneously the hexagons and spheroids contained in

Fig. 10. Cell division leading to the formation of the thread cells (TD). X 2200.

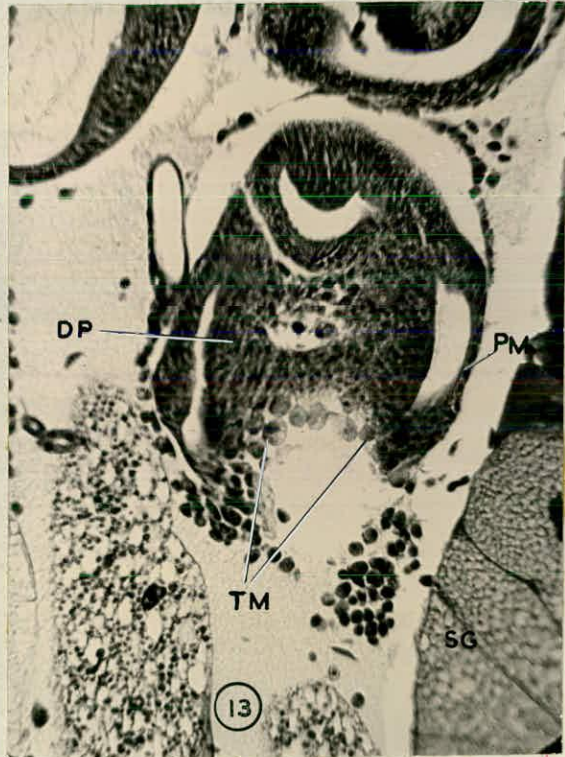
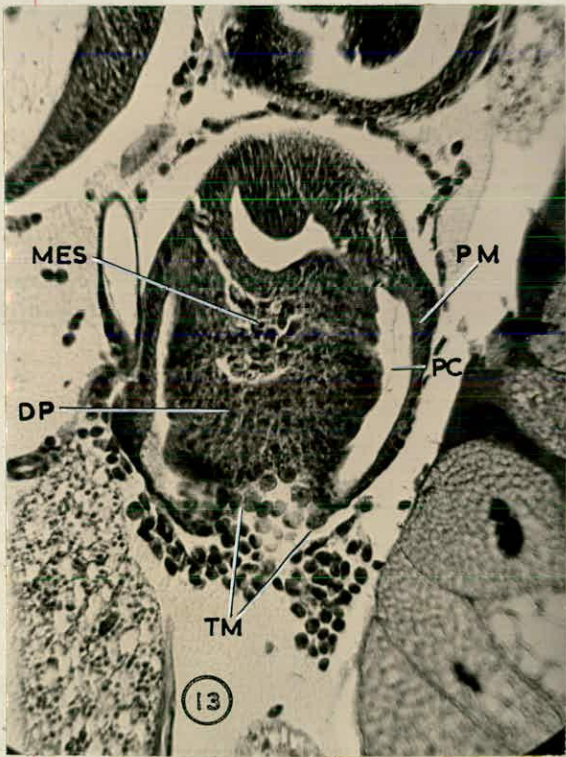
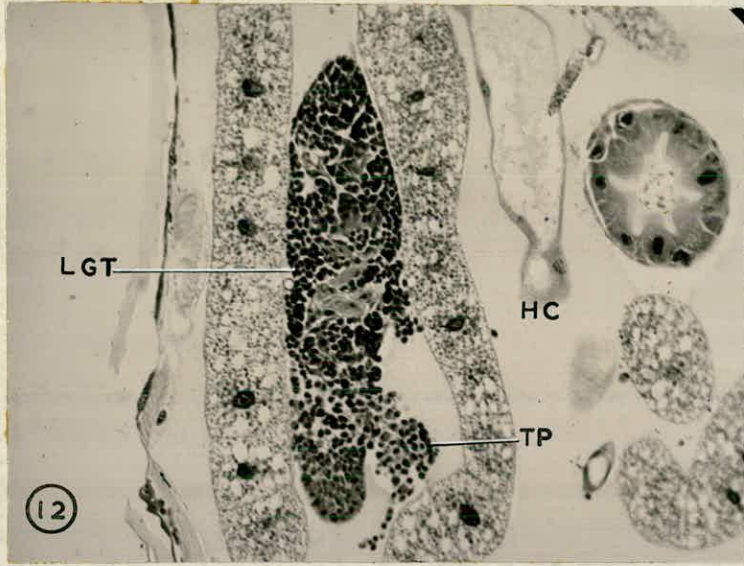
Fig. 11. ^{type of} The same cell division as illustrated in Fig. 10. Notice a thread cell precursor completely surrounding its sister rounded cell. X 2200.



the gland fall out through the ruptured epithelium and are set free in the haemolymph (Fig. 12). The cell proliferation which had led to the rapid increase in size of the lymph glands continues both in the cells which remain within the gland and those which are released into the haemocoel. This excessive proliferation justifies one in referring to the tissues as tumours. The original lymph glands may now be referred to as the primary tumours while, as we shall see, the released cells form secondary growths and undergo processes of true metastasis.

Primary aggression: the imaginal disc.

The hexagons and spheroids released from the lymph glands and which comprise the mixed-cell type tumour tissue, do not move at random but are transported in a definite direction by the circulation of the haemolymph. They are, to begin with, moving anteriorly into the region which is crowded by the imaginal discs. The released tumour cells not only rapidly accumulate around these discs, but actually invade and penetrate them. There is no particular direction of this invasion but it frequently occurs through the peripodial membrane which is a relatively delicate structure. Passing through this the tumour cells accumulate within the cavity of the disc and proceed to invade both the disc proper and its mesoderm. Fig. 13, a and b, show two sections through a disc which is surrounded and



partly penetrated by the released tumour cells. Fig. 14a, b and c, show three sections through another imaginal bud in which the peripodial membrane and other pods have been very largely destroyed by the invading tissue.

It is worthy of note that no case has been seen in which all the imaginal discs are invaded simultaneously. The dorsal set of buds and particularly the eye-antennal complex are the first to be attacked, probably because they lie nearest to the blood-forming organs. It is only later that the tumour tissue may spread to the ventral set of buds.

The infiltration of the imaginal discs continues until, in the 8 days old larva, they become completely necrotic and no longer show any specific characteristics. Fig. 15 shows a medio-sagittal section which passed through the eye-antennal bud and the first and second pair of leg buds. The first of these is the most heavily infected and shows the septum separating the eye and antennal portions completely severed. Fig. 16 is another section cut dorso-lateral and shows the condition of the buds at later stages in the tumour attack. In the final stages the necrotic imaginal discs show no pattern of organisation whatever but are mere masses of tumourous material.

Metastasis

In vertebrate malignant growths metastasis or the

Fig. 14. a, b and c represent the same infected imaginal disc in three adjacent sections. a shows complete infiltration of the central part of the disc, b shows partial necrosis of the central part while c shows complete necrosis of the imaginal bud in that area. TM - tumour cells; IG - imaginal disc; TC - flattened tumour cells passing in between the fat bodies and the imaginal bud. X 300.

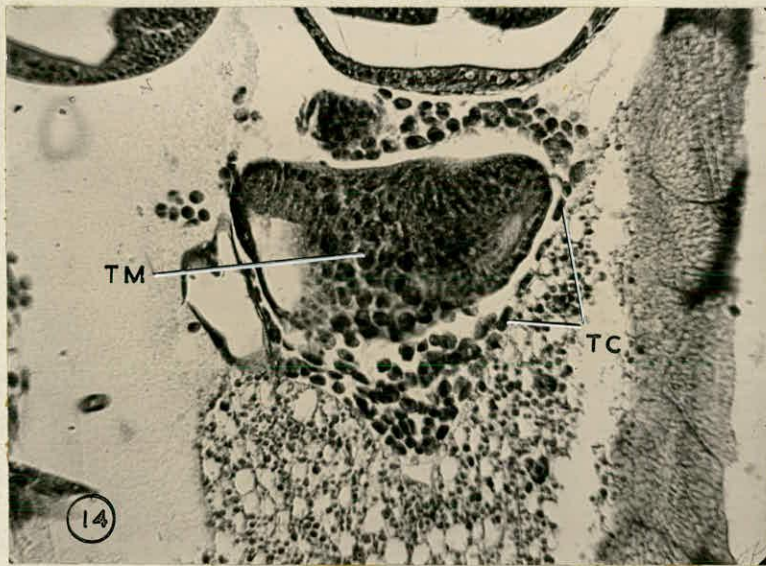
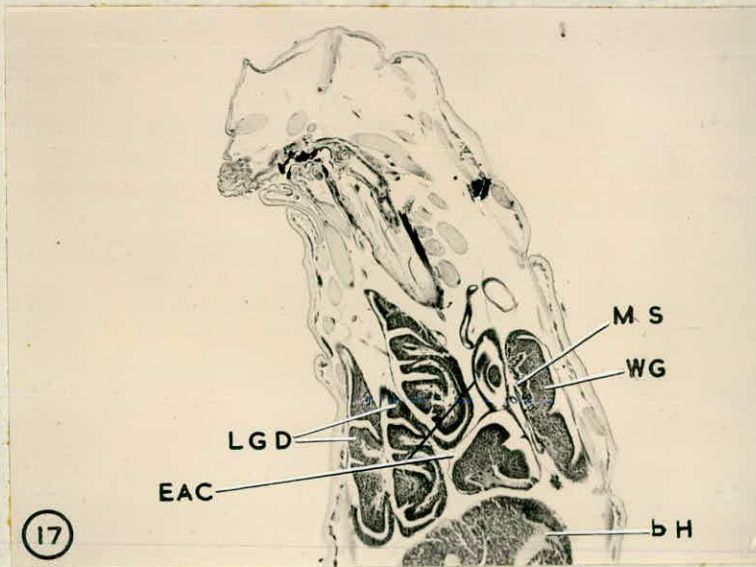
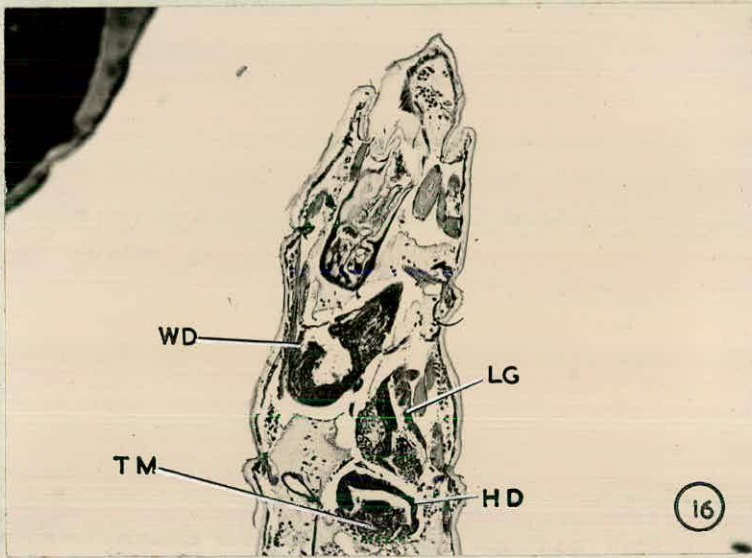
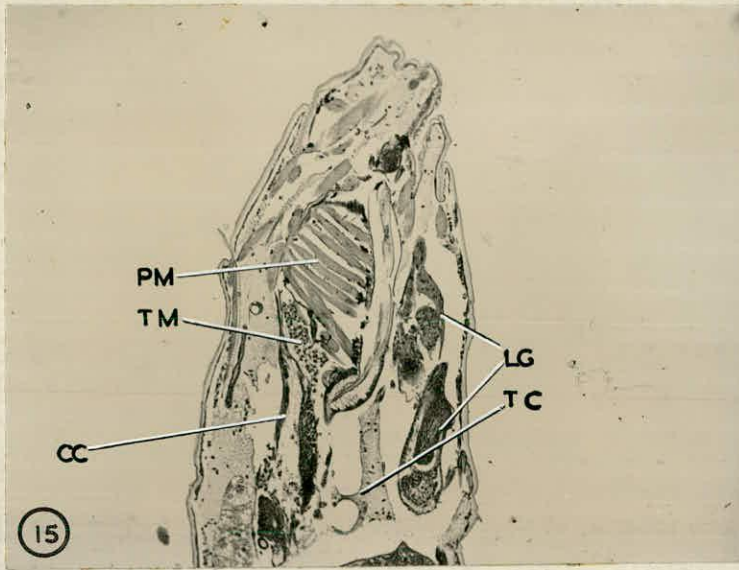


Fig. 15. A medio-sagittal section in the cephalic portion of a late malignant larva demonstrating necrosis of the imaginal buds. PM - pharyngeal muscles; TM - tumour cells; CC - cephalic complex; LG - leg disc; TC - trachea. X 70.

Fig. 16. An oblique section passing through necrotic lateral ^{series} of imaginal buds. WD - wing bud; LG - leg disc; HD - haltere disc; TM - tumour cells. X 70.

Fig. 17. Sagittal section in a late third instar wild-type larva. WG - wing bud; LGD - leg discs; EAC - eye-antenna complex; MS - mesoderm; BH - brain hemisphere. X 80.

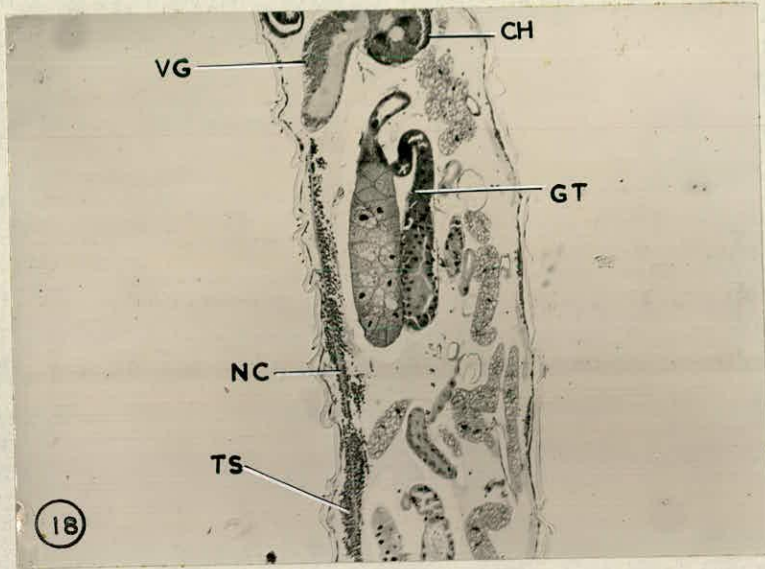


transportation of tumour cells to secondary sites is a very definite process and is carried out either by the lymphatic or blood stream. In Drosophila the haemolymph flows from the dorsal aorta to bathe the anterior region. It then moves backward along the visceral sinus from which it is taken up again in the pericardial sinus to the heart. There is, in addition, a narrow neural sinus lying along the ventral surface of the body and roofed dorsally by a neural septum which lies just above the nerve cord (a fuller description of the circulatory system is given later, see page (57). According to this we might expect that metastatic growths should be distributed from the lymph glands first towards the anterior, where we have them in the imaginal buds, and later towards the posterior.

The facts bear out this expectation. The tumour cells accumulate particularly around the ventral nerve cord and spread towards the posterior. Figs. 18 and 19 show a specimen in which the nerve cord is heavily loaded with tumour cells. It is not clear whether the neural septum remains intact but it appears probable that it has been destroyed so that the nerve cord is exposed in the visceral sinus. It is probable that the destination of the metastatic growths is mainly determined by the circulating haemolymph, and that the nerve cord serves only as the substratum along which they move.

Fig. 18. Metastasis along the ventral nerve cord. CH - cerebral hemisphere; VG - ventral ganglion; GT - gut; NC - ventral nerve cord; TC - metastatised tumour tissue. X 50.

Fig. 19. A portion of the nerve cord of Fig. 18 at a high magnification. NC - nerve cord; TM - transported tumour tissue. X 780.



At this stage, that is fairly soon after the release of the tumour cells from the lymph glands, these cells are never found free in the central body cavity, but occur only in the three main foci characterised by malignant activities, namely -

- (a) the lymph glands themselves (primary tumours),
- (b) the imaginal buds (the primary centre of aggression, and
- (c) the ventral nerve cord.

In all these three localities the neoplasm can be seen to be made up of the two kinds of cells, namely hexagons and spheroids.

Tumour cells are metastasised to attack the fat bodies.

Secondary attack: the fat bodies.

The tumour cells continue spreading along the ventral nerve cord until they reach the posterior end of the body. They then accumulate as a mass of free cells in the body cavity at the caudal end of the haemocoel. This region contains the main larval fat bodies and these bodies are the first to receive the nerve cord spread. Fig. 20 shows a heavy collection of tumour cells in the neighbourhood of the fat bodies, while in Fig. 21 the invasion of these bodies has begun and tumour cells can be seen within them. The invasion continues until the fat bodies are completely infiltrated. The invading tumour cells often arrange themselves in

Fig. 20. Collection of the tumour tissue in the caudal haemocoel for attack on the fat bodies. TT - tumour tissue; GD - genital disc. X 130.

Fig. 21. A stage later than that in Fig. 20, showing early invasion of fat bodies. TM - tumour cells invading the fat bodies (FB). X 70.



rosette patterns around the fat body vacuoles, giving rise to pictures which recall those found in some human brain tumours (Fig. 22). In later stages these rings of cells become quite enlarged in size (Fig. 23). At late stages of the attack (Fig. 24) the fat bodies are scarcely any longer recognisable (compare control, Fig. 25). The fat body nuclei which normally contain chromosomes in the form of polytene threads, are broken down to deeply stained granules (Fig. 24). The nuclear membranes are no longer identifiable.

Active cell proliferation continues in the malignant tissue and the infiltrating neoplasm spreads in all directions. Fat bodies other than those at the posterior end of the larva are eventually attacked.

Necrosis

The eventual necrosis of the fat bodies shows some characteristic features. In Fig. 26 one sees a necrotic fat body from the anterior thoracic region. There is little to be seen but some ill-defined remnants of tissue containing free tumour cells. Fig. 27 illustrates the condition in a fat body from the posterior, that is to say from the region which first receives the invading tissue which spreads along its nerve cord. It shows the tumour cells have become altered so that they form quite large black bodies which are attached to or inside the fat tissue itself. The formation of such melanised structures is discussed in more detail later.

Fig. 22. Rossets of tumour cells within the invaded fat bodies.
Notice the cells arranging themselves in a peculiar manner
around the fat body vacuoles. X 800.

Fig. 23. Increase in size of the tumour cell rings in the fat
bodies. X 260.

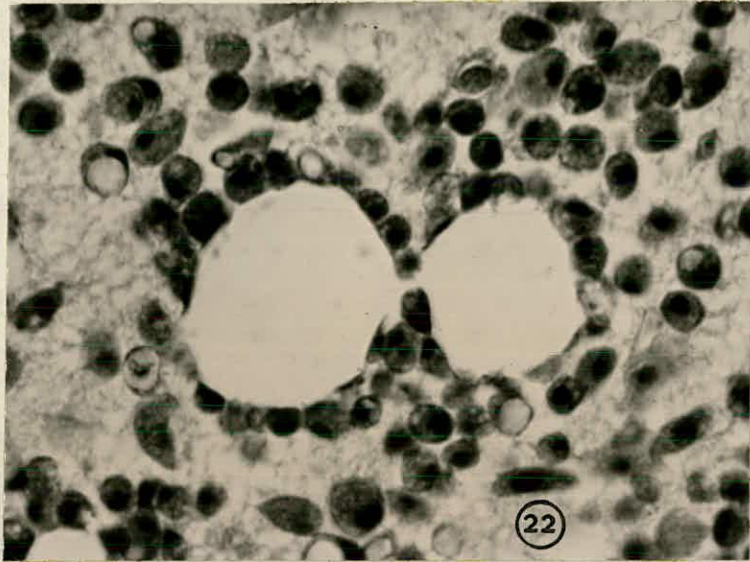


Fig. 24. Complete infiltration of the fat bodies. TM - heavily crowded tumour growths; NU -remains of nuclei. X 240.

Fig. 25. Horizontal section in late third instar wild-type larva passing through the posterior abdominal region. FT - fat bodies; SP - spindle cells; HI - hind gut; RS - rounded spheroids. X 140.

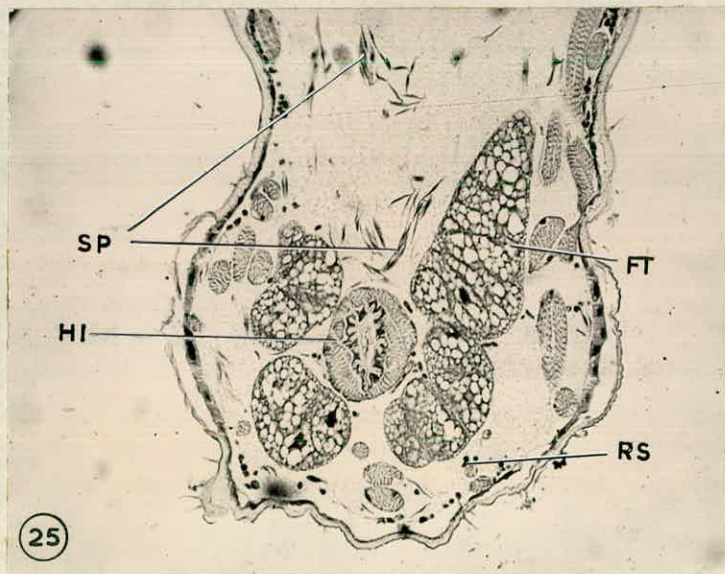
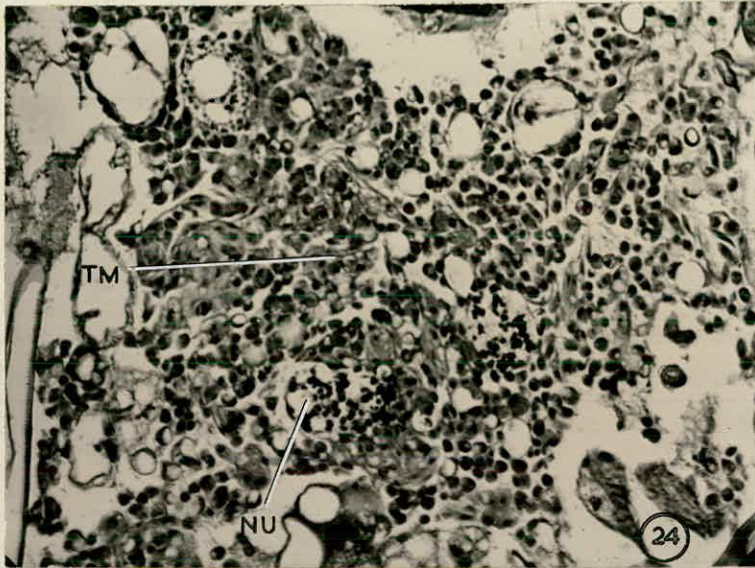
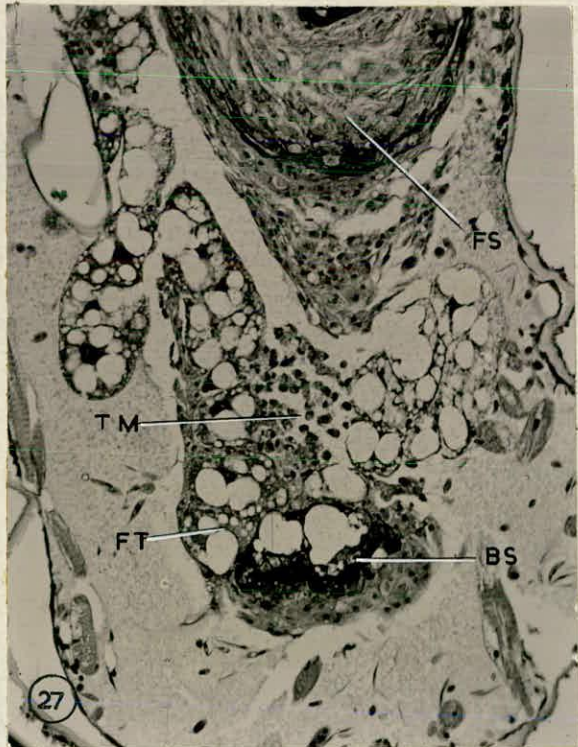
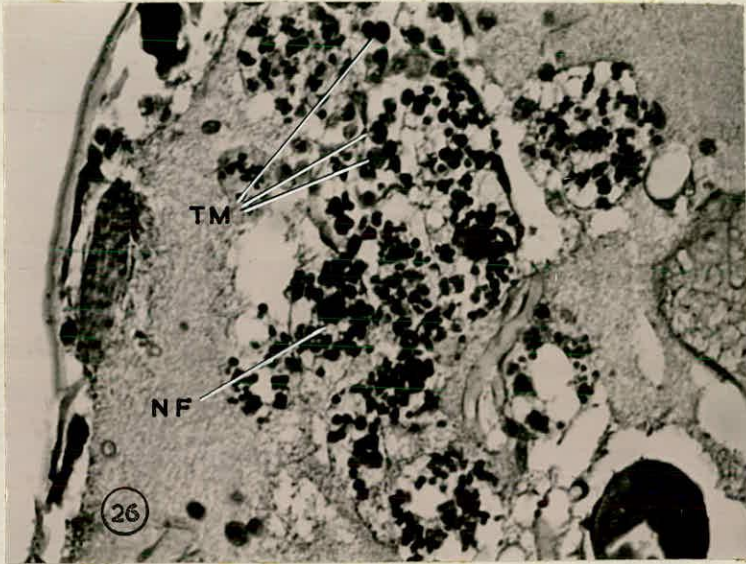


Fig. 26. Necrosis of anterior fat bodies. TM - tumour cells;
NF - necrotic fat masses. X 350.

Fig. 27. Necrosis of posterior fat masses. TM - tumour cells;
FT - fat bodies; BS - black bodies formed inside the
necrotic fat bodies; FS - free melanised structures. X 600.



Incidence of secondary aggression

Larvae of the type just described, which show not only primary aggression on the imaginal buds but also secondary aggression on the fat bodies with metastasis along the ventral nerve cord, die without ever forming puparia. They are generally obtained from crowded culture bottles and represent the strongest grade of expression of character. Throughout the stocks investigated, metastasis is of frequent recurrence, but secondary growths fail in the majority of cases to establish themselves. This is because antagonistic reactions occur which tend to neutralise the tumour tissue. These will be discussed later in connection with the anti-neoplasm activities (page 33). It is, of course, well known that in some vertebrate tumours secondary foci are often unsuccessful in establishing themselves. For example, muscles may be flooded with cancer emboli and yet a secondary growth in muscle is the rarest occurrence. Tumour emboli in the lungs usually fail to implant properly and to develop into metastatic growths as they become coated with fibrin and die out.

Other abnormalities characteristic of the malignant phenotype.

Most of the abnormalities noticed in the lethal larvae are directly connected with the tumour growth.

For instance the ventral nerve cord may eventually be completely destroyed or reduced to mere fragments scattered in the body cavity. The tracheae, other than the two main lateral trunks, are frequently severed and broken. Studies on the behaviour of the salivary glands and alimentary canal are being carried out. There is, however, another rather striking abnormality, the connection of which with the primary tumour is not so direct. This involves the testes.

Wild-type testes.

In a late third instar larva of wild-type Drosophila the testis is regionally differentiated into four major groups of cells (Geigy 1931, Geigy and Aboim 1944, Aboim 1945, Gloor 1943). Cephalically there is a small cluster of apical cells, of a very small size compared with the spermatogonia which follow in order. Behind these the primary spermatocytes successively increase in size as the caudal end of the testis is approached. At the hindmost extremity there lies a group of terminal cells of a slightly bigger size than the apical ones. No further-advanced germ cells have been reported in literature on this subject.

In homozygous lethal larvae the late third instar testes are normal. In 5 days old testes, however, some unidentified cells from the vicinity of both spermatogonia and terminal cells become very pronounced as they acquire a peculiar shape and react vigorously to basic

stains (Fig. 28). Each such cell is more or less triangular, with a large nucleus near one end and a marked basal extension; some of them seem at least binucleated (Fig. 29). These testis tumour cells invade the adjacent germ cells, particularly the primary spermatocytes. They proliferate and increase in number so that 6-day old testes show a considerable number of them, with very frequent late division configurations (Fig. 28 and Fig. 29). The tumour cells persist in 7-day old larvae and the invaded areas are becoming necrotic (Fig. 30). Necrotic areas are not, as shown in the Fig. , restricted to the caudal portion of the testis, but can occur anywhere, depending on the extent of invasion. Some preparations showed the whole germ tissue necrotic. The necrotic areas appear homogenous, and react negatively to haematoxylin. The vacuoles included in them represent extremely cytolysed tumour cells while the areas between are filled with coagulated testis tissue. While this pattern regularly appears in all lethal individuals, some larvae showed in addition a feature which, in wild-type individuals, is restricted to pupal stages. The primary spermatocytes divided to secondary then to spermatids. This was shown by the occurrence of division cysts of four cells each. Fully mature sperms eventually make their appearance in the lethal larval testes. They are initially quite normal, but later become abnormal as illustrated by Fig. 31, where the

Fig. 28. A section passing through the cephalic portion of a lethal testis showing TTM - testis tumour cells; SSP - secondary spermatocytes. X 770.

Fig. 29. Multiplication of testis tumour cells. DC - dividing tumour cell. X 550.

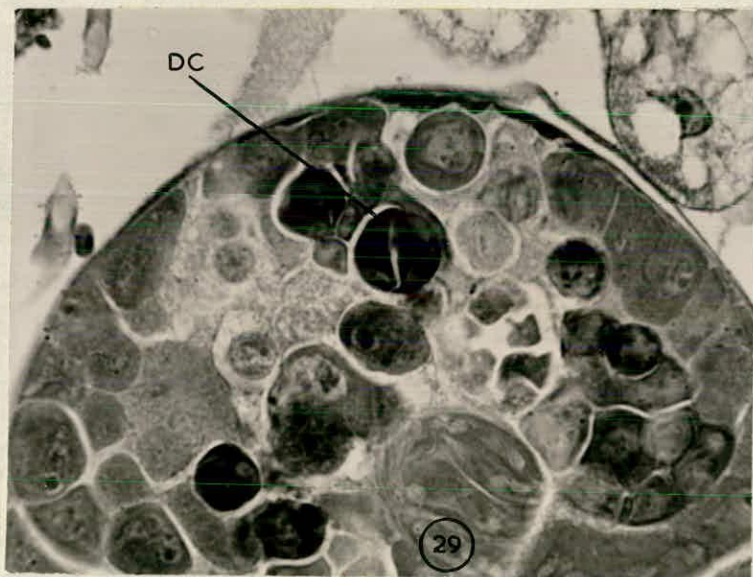
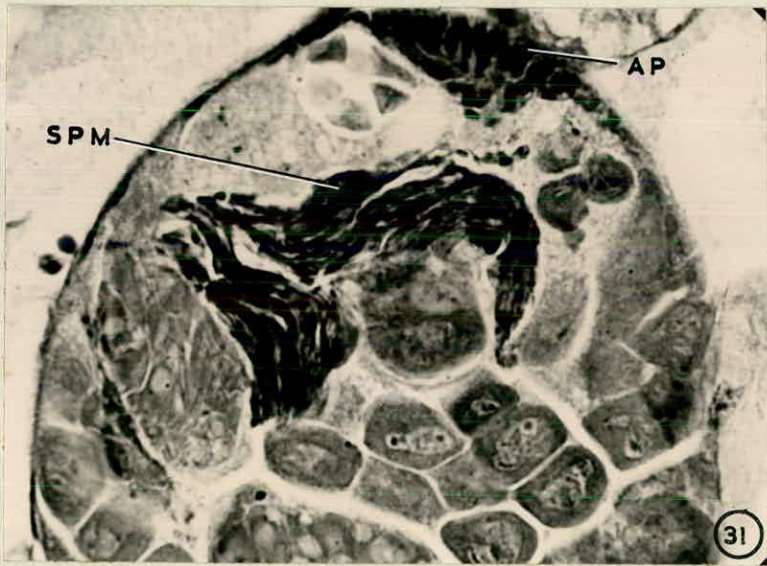
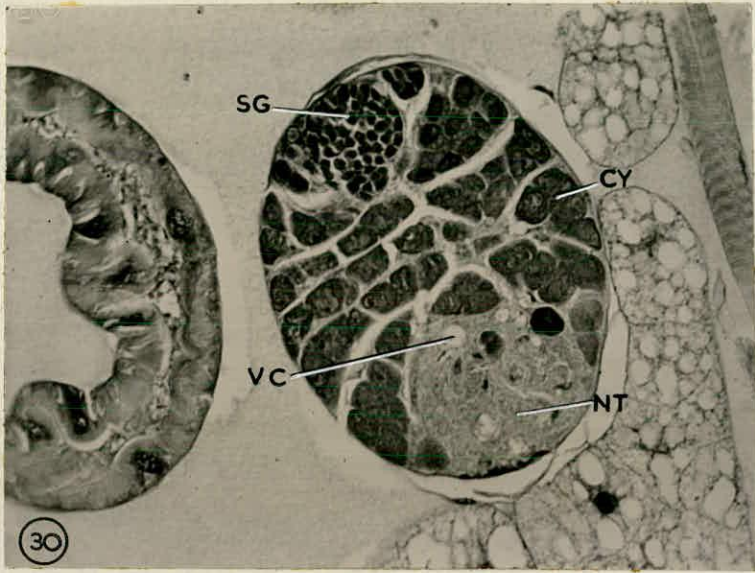


Fig. 30. A section in 7 day old lethal testis illustrating necrosis. NT - necrotic areas; VC - vacuoles in place of the dead tumour cells; SG - spermatogonia; CY - secondary spermatocytes. X 270.

Fig. 31. A section in 7 day old lethal testis of another specimen showing dead sperms (SPM); AP - apical cells. X 770.



identified sperms, which are long and coiled as is normal, stick closely to each other and fragment to pieces. Most interesting is their affinity towards basic dyes, which is relatively high compared with normal pupal or imaginal sperms, which are negative to the Feulgen-reaction. In later stages these larval sperms lyse completely.

Anti-neoplastic activities.

In different individuals of the stock investigated, which were, of course, by no means isogenic, there was considerable variation in the degree to which the tumour was developed. Some of this variation may well have been due to differences in the strength of the tendencies which act to favour the growth of the tumour. Much of it, however, is brought about by the greater or lesser expression of tendencies which act to inhibit or suppress the tumour tissue. These may be referred to in general as "anti-neoplastic activities". We shall first describe two related but slightly different types of activity which tend to suppress tumour growth in its early stages and later (page 39) a third type which acts at a later stage.

As was described above, the normal lymph glands contain three different types of cells of which only two types - the hexagons and spheroids, with some assistance from the new type of thread cells - give rise to the malignant tissue. The role of the third type,

the platelets, during these processes has not yet been referred to. Transfer to malignant conditions is a phenomenon where two events have been observed to occur simultaneously. While hexagons and spheroids prepare for aggression, the platelets prepare for the function of defence by antagonising the newly emerged neoplasm. It is the latter cells, whose function is described below, that sometimes played a leading role in anti-neoplasm activities.

Before dealing with the anti-neoplastic activities, it is necessary to describe some of the types of cells to which the platelets can give rise. These will be described in terms of the type of cell division by which they are formed. These division processes are again not of a conventional type but occur in cells which are already somewhat differentiated and which are proceeding to further differentiations. Although it is not possible to watch the divisions occurring in living material, it is believed that the deductions which have been made in the study of numerous sections give a reasonably accurate account of the processes occurring.

Normal platelets are triangular cells with centric nuclei. They give rise to three main types of cells, all of which perform similar functions which may roughly be described as that of encapsulating the hexagons and spheroids of the tumour. These types of cells are as follows:-

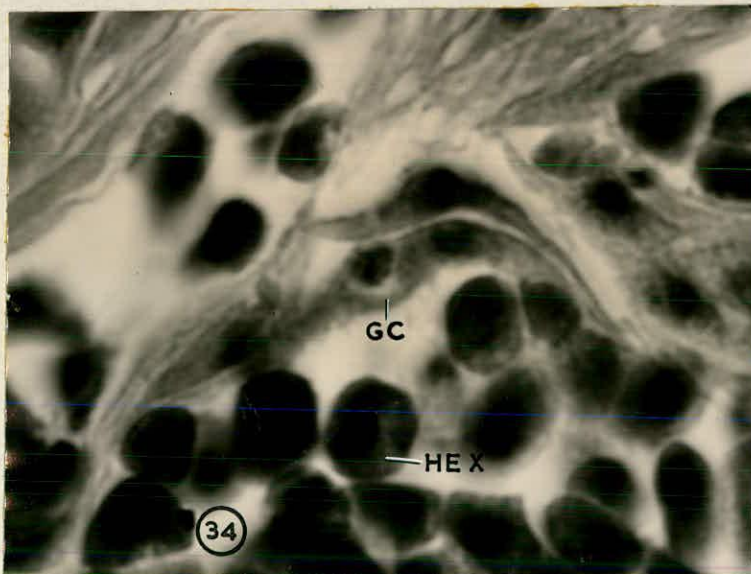
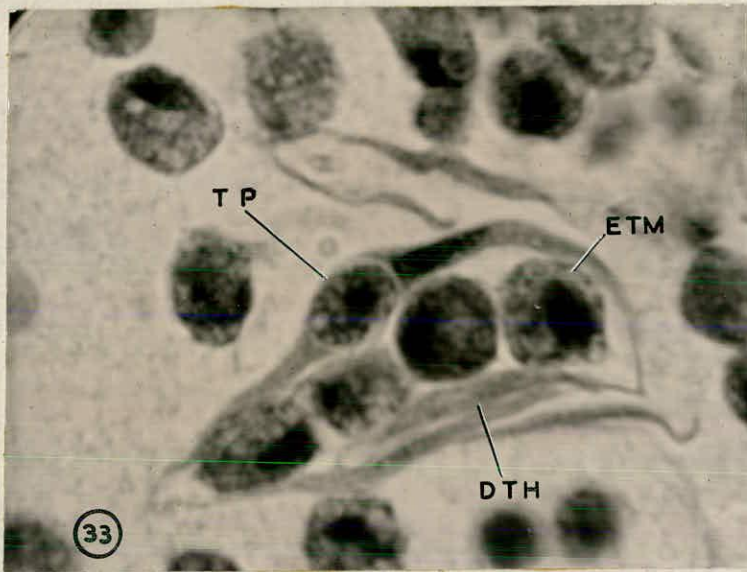
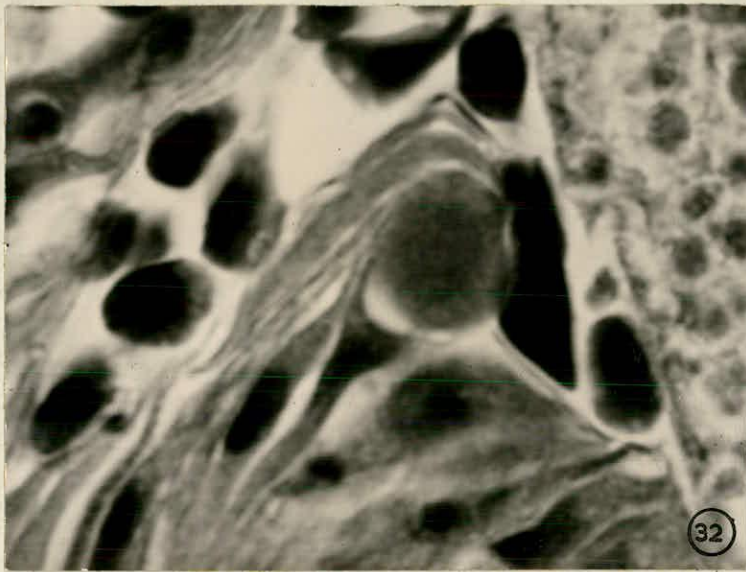
1) Giant cells. A platelet cell may increase considerably in size, the narrow portion of the triangle becoming very elongated. A cell at this stage appears to consist of a main, somewhat rounded, body containing the nucleus and a long tail-like extension. The nucleus then migrates to the intermediate zone where the rounded portion is just narrowing to join the filamentous tail. A crescent-like cleavage plane may then appear, which separates the rounded portion of the cell from the tail, which is somewhat Y-shaped, consisting of a long shaft which is continued anteriorly as two lateral processes (Fig. 32). The nucleus usually divides before this cleavage takes place so that both sister cells are nucleated. In some cases, however, no nuclear division takes place and the single nucleus remains in the Y-shaped tail portion situated at the junction of the two short, finger-like processes. The fate of the rounded daughter cells is not clear. Elongated Y-shaped cells are referred to as Giant cells.

2) Tripartite cells. A platelet may increase in size and elongate in two opposite directions so that it appears as a central oval portion extending on both sides into two long tapering arms (Fig. 33). What appear to be three nuclei make their appearance. Two of them migrate to the thicker portions of the two extensions, while the third remains in the central oval portion. A deep suture appears round

Fig. 32. Cell division leading to formation of giant cells. Notice anucleated spherical cell just separated from a young giant cell. X 2200.

Fig. 33. A tripartite cell (TP); ETM - encapsulated tumour cell; DTH - dead thread cells. X 2200.

Fig. 34. Late stages in the formation of binucleated giant cells (GC); HEX - binucleated hexagons. X 2000.



the central part so that the structure appears to have three rather distinct components - two identical arms continuing with each other through a ring of cytoplasm which embraces another rounded central mass. These three parts do not separate from one another. The cells showing this structure have been called "tripartite cells". They are found in the body cavity in connection with metastatic growths and other frequent occurrences.

3) Binucleated giant cells. A platelet increases in size and flattens extensively, the central part eventually becoming reduced (Fig. 34). The nucleus divides into two, but the two daughter nuclei continue to lie near one another. The cytoplasm remains undivided and the result is a binucleated cell. The figure shows two such cells with pointed, whip-like ends, and two nodes, one larger than the other, representing the nuclear regions. These binucleated giant cells, like the tripartite cells, are released into the haemocoel.

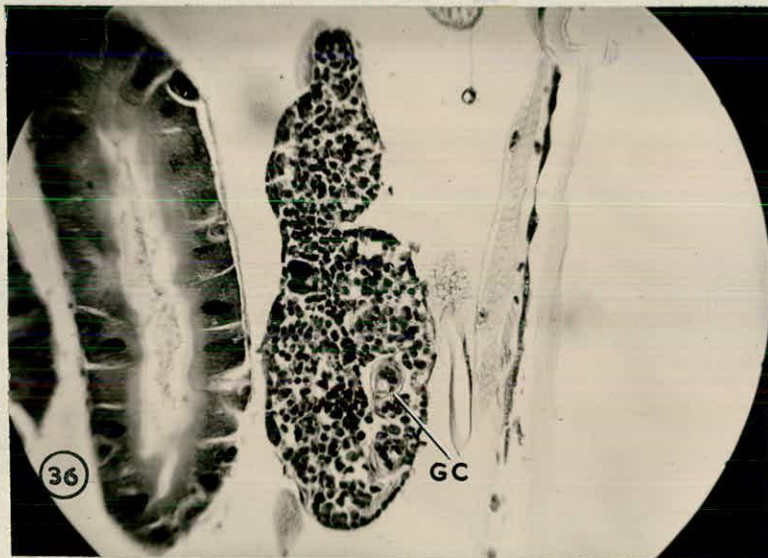
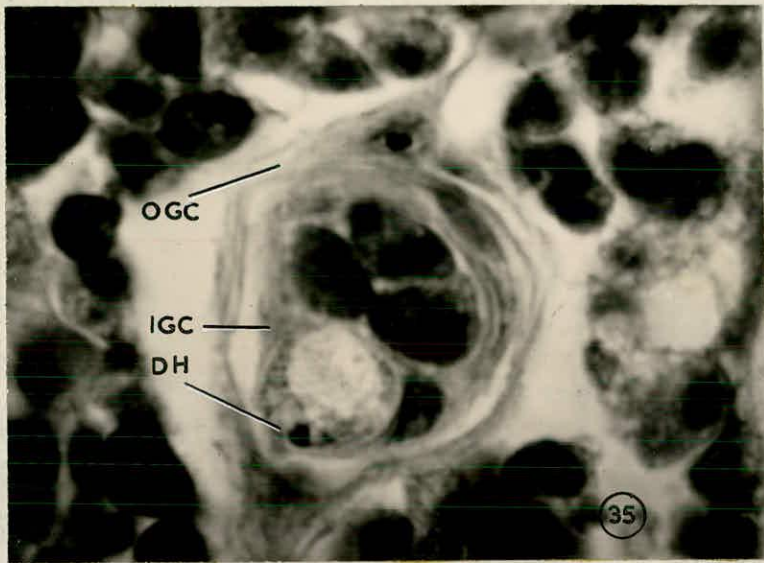
These types of cells derived from the platelets act so as to inhibit the tumour growth by encapsulating the tumour-forming hexagons and spheroids. This type of activity can take place either within the lymph glands and at a fairly early stage, or at a later stage in the haemocoel, during the metastasis of the tumours. In the former case the active agents are the

giant cells. They exhibit two rather different types of encapsulating activity, shown as follows.

Type 1. Only in two, out of about four hundred 5-day old specimens examined, were the lymph glands found to be degenerate and the body cavity was found free of tumour cells. These lymph glands contained a number of small patches of clustered and encapsulated tumour cells. Fig. 36 shows one of these capsules which is taken at a high magnification in Fig. 35. The investing cells are giant cells, such as that shown in Fig. 32, which have been slightly modified by a still further elongation of their two projecting extensions. The cells enclosed in these capsules seem to consist of always solely hexagons. This selectivity in the encapsulation is very remarkable and it is not clear how it is achieved. The encapsulated hexagons seem to degenerate rapidly. One of those shown in Fig. 35 has already died and exhibits a vacuolated cytoplasm. The lymph gland shown in Fig. 36 showed only one such giant capsule. However, several unenclosed patches of dead hexagons were abundant. It is possible that the investing giant cells are freely motile and that after enclosing one group of hexagons for long enough to cause their degeneration, they move on to enclose other cells. This is perhaps a rather unlikely suggestion. It is in any case noteworthy that the proliferation of the spheroids in these glands is also suppressed, although cells of this type never seem to be enclosed into capsules. The inhibiting action of the giant

Fig. 35. The giant capsule in Fig. 36 taken under a high magnification. IGC - inner giant cell; OGC - outer giant cell; DHX - dead hexagon. X 2000.

Fig. 36. Aggressed primary tumour showing a giant capsule (GC).
X 320.



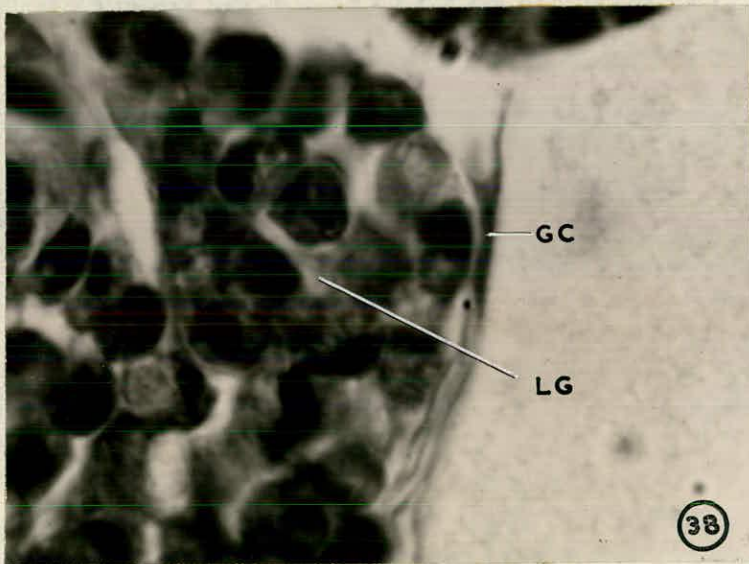
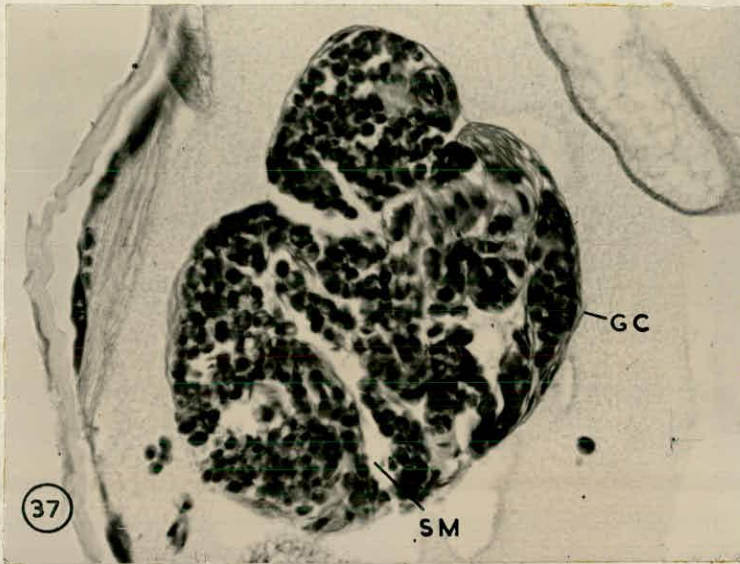
cells therefore seems to be something more than a mere mechanical result of the enclosure of the hexagons.

Type 2. In several other specimens, cells derived from the platelets surrounded and enclosed not a few tumour cells but whole tumour masses. In these cases the platelets divided more frequently to give rise to "giant cells" which were quite small in size. These arranged themselves around the whole mass of the tumour to form a strong encapsulating sheath. Fig. 37 shows an example of this, while in Fig. 38, a higher magnification, one can see that both hexagons and spheroids are present within the capsule, while the capsule-forming giant cells can also be seen. Proliferation of the tumour cells continues within the capsule so that the lymph glands enlarge but the wall does not rupture and the cells are not liberated into the haemocoel.

The encapsulating activities which occur at a later stage after the tumour cells have been replaced into the haemocoel, are much less effective. They occur particularly among the cells which are moving along the nerve cord and hinder, to some extent, the invasion of the posterior fat bodies. They are carried out by the tripartite and the binucleated giant cells which have been described above. Fig. 33 is an enlarged portion of Fig. 21 and shows a tripartite cell combining with some thread cells (probably already

Fig. 37. A primary tumour encapsulated by tiny giant cells (GC);
SM - stroma. X 400.

Fig. 38. A highly magnified portion of the body wall of the
primary tumour shown in Fig. 37 illustrating the nature of
the encapsulating cells. GC - tiny giant cell; LG -
lymph gland tumour cells. X 2000.



dead) to encapsulate a group of four large tumour cells. Such pictures are frequent amongst the crowds of hexagons and spheroids invading the fat bodies. In such groups the encapsulated cells may have disintegrated while the enveloping ones remain apparently healthy. It is not clear whether the latter would also die shortly after the enclosed cells or whether they can move on to encapsulate still further healthy tumour cells. The capsules formed by the binucleated giant cells are essentially similar to those produced by the tripartite cells. An example is seen in Fig. 46.

A third type of anti-neoplasm phenomenon.

In a considerable number of the investigated lethal larvae, the tumours had proliferated freely and tumour cells in abundance were released in the body cavity, where some started to invade the imaginal discs and others established themselves along the ventral nerve cord. However, at the beginning of metastasis a very pronounced phenomenon occurred in the tumour tissue in its three centres of proliferation. Fig. 43 is taken from a semi-lateral section cut through the anterior region of the ventral nerve cord in such a specimen. The scattered fragments represent some of the main nerve fibres. The striking point is that the hexagons have become melanised and appeared as big dots of a reddish tint easily distinguished from the other component of the tumour tissue - spheroids - which are

much smaller in size. Spheroids at this early stage of the anti-hexagon reaction still appear, histologically, to be malignant. However, in older larvae in which such changes had taken place at an earlier stage, the growths which were being metastatised have become anchored in situ (Fig. 44). Hexagons have disintegrated completely, and vacuoles taken their place; spheroids are clumped around the nerve cord with their nuclei broken down and cytoplasm vacuolated. The same condition prevailed in the tumours as well as the primary growths. Attack on the imaginal discs did not proceed to any considerable extent, since the malignant tissue was antagonised at an early stage of its invasive activity. However, imaginal disc mesoderm showed the abnormal phenotype to be described later (page 46). Due to failure of metastasis, individuals falling into this category show the visceral sinus and the caudal portion of the ventral nerve cord free of tumour cells.

A large number of 6½-day old larvae showed metastasis successfully carried out, yet the fat bodies were not affected. The tumour cells crowding the haemocoel, particularly the area of the posterior fat masses, aggregated to form big clumps of tumour tissue, and a great number of them acquired a spindle shape. The primary growths however appeared abnormal, but the imaginal buds had been partially or completely

infiltrated. This is demonstrated by Fig. 45, where tumour growths in a cephalic and wing bud have destroyed the mesoderm.

Test of malignancy diagnosis

As an incidental observation, lethal larvae were found to respond in a peculiar manner towards high temperature shocks. Applying Bouin at 85°C as a routine fixative, the tumour cells transform immediately to dark spots which are quite easily visible with the naked eye. When treated larvae are investigated under a low power binocular, these dark dots which represent the fixed tumour cells are found not to be distributed at random. In fact, the extent of the distribution of dotted areas is identical with the progress of the neoplasm. The reaction is most pronounced and striking during metastasis. Fixed malignant larvae at early stages show their blood-forming organs heavily dotted. This pattern continues fronto-dorsally, representing the course towards the imaginal discs. The extent of the metastasis along the nerve cord is directly represented by the length of the area which revealed the spotting: in some cases only a short distance reacted positively; in others the area surrounding the whole length of the ventral nerve cord was heavily crowded with the reactive tumour cells. Larvae, where metastatic processes have been completed, showed the dark spotting scattered all over, indicating

the onset of secondary aggression. Cases which have overcome their neoplasm by the formation of "black bodies" (page 43) are negative to the treatment, while those which have not developed the melanised bodies, and yet are negative to the test, showed, on microscopical investigation, that they had already antagonised the malignant tissue. However, a positive reaction to this test, as has been confirmed by histological methods, is a reliable indication of a blood-forming organ carcinoma. No other tumour stock has been found which might help to validate this diagnostic criterion on a wider variety of material. However, subsequent work in the second part of this thesis has supported its diagnostic value (page 13).

The reaction was not specific to hot Bouin. Boiling water, which has been frequently used as a satisfactory fixative for *Drosophila* material, secured the same results. The causative agent is, therefore, the high temperature shock and not any particular chemical molecule related to the fixative. This test is consequently given the name high-temperature shock reaction, abbreviated as H.T.S. reaction. Microscopic investigations showed that the black spotting is not due to a pigment precipitation, since cell structure in materials treated for the test showed no significant difference from those fixed with cold agents.

This reaction could be of the utmost value in Drosophila tumour work, as any lethal stock suspected of malignancy could be simply tested for it without the application of the exhausting technique of histology peculiar to Drosophila larvae. The nature of the reaction however must await explanation until further studies on the cytochemical properties of Drosophila tumour cells are carried out.

Formation of "black bodies"

After primary and secondary aggression, or the former alone, are completed and have resulted in necrotic tissues, the tumour cells undergo processes by which black bodies (melanotic tumours) are produced. These structures are formed inside the fat bodies following successful secondary growths, or more frequently in the haemocoel. In the former case they appear microscopically as melanised capsules surrounding the fat body vacuoles. The inter-capular^s space is occupied partly by clumps of spheroids and partly by fine melanised strata. The black bodies free in the haemocoel are oval and are formed by successive layers of tumour cells which are precipitated on their surface. The external layer is composed of a network of melanin which usually extends throughout the thickness of the black body in irregular sheets. In section the bodies therefore appear to be cut up into spaces of various sizes. However, the processes

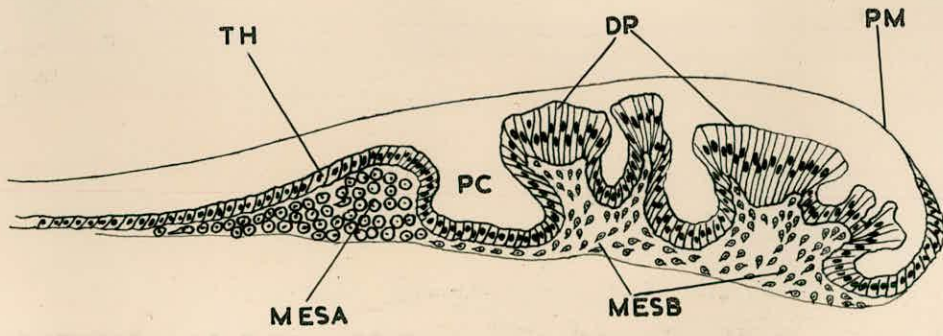
by which the black bodies in the haemocoel and in the fat masses are formed are identical. The only difference lies in the site of formation which depends on the amount of tumour tissue and on the occurrence of secondary aggression. Lethal malignant larvae in which the latter occurred showed them in both localities. In other stocks they are more frequently found only in the haemocoel.

Shortly before the death of the larvae both the hexagons and the spheroids composing the tumour show remarkable changes in their morphology. These primarily concern the nuclei which lose their staining characteristics and sometimes break down into granules. The cytoplasm becomes vacuolated. A large number of spheroids transform into spindle-shaped cells and intergrades representing a gradual transformation from rounded to a spindle configuration are abundant. A fully formed spindle cell is very narrow in shape and has long tapering ends. Spheroids which are not transformed in this way aggregate to form clumped masses which are later enveloped by strata of spindle cells (Fig. 47). A compound structure is eventually formed with spheroids comprising its main volume and spindle cells covering the surface. These processes are repeated as the black bodies increase in size. This increase usually proceeds rapidly. A black body may reach several times its original size in a relatively short period.

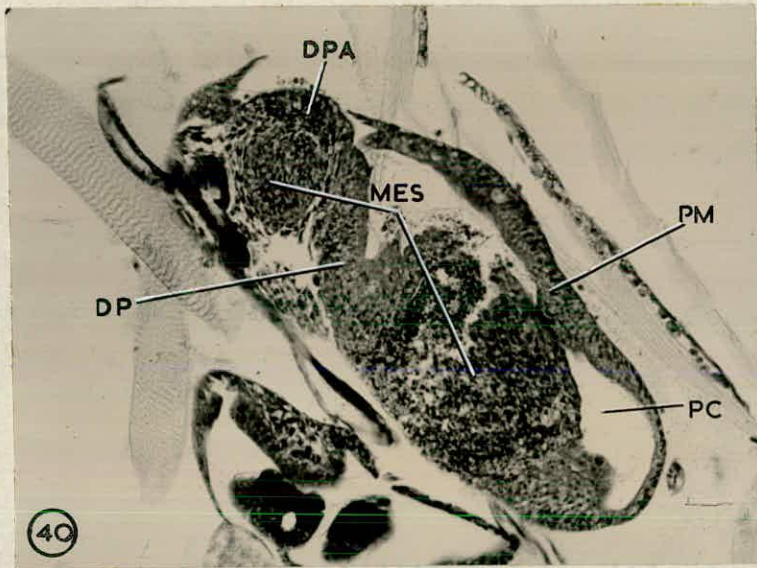
Fig. 39. A diagrammatic representation of late third instar wild type imaginal disc showing sub-differentiation into A and B segments (Text). MESA - mesoderm of segment A; MESB - mesoderm of segment B; DP - segmented disc proper; PC - peripodial cavity; PM - peripodial membrane; TH - imaginal disc epithelium which forms the thoracic integument.

Fig. 40. A wing bud from larvae with antagonised tumours of Figs. 37 and 38 showing the rupture of the disc proper portion of region B (Text). MES - mesoderm; PM - peripodial membrane; PC - peripodial cavity; DP - disc proper; DPA - ruptured disc epithelium of A region. X 200.

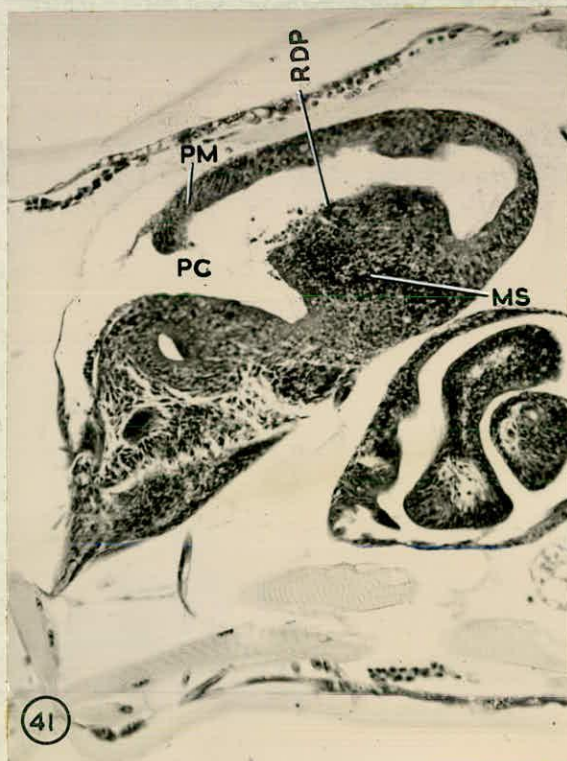
Fig. 41. A slightly more advanced stage than that in Fig. 40, showing complete rupture of the disc proper in the area of the proliferated mesoderm. RDP - place of ruptured disc proper; MS - mesoderm; PM - peripodial membrane; PC - peripodial cavity.



39



40



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Fig. 42. A wing bud showing the difference in the behaviour of the mesoderm of segment A and B (Text). MS - mesoderm of A segment; PC - peripodial cavity; DP - disc proper; PM - peripodial membrane; AMP - abnormal mesoderm proliferations falling in the disc cavity. X 300.

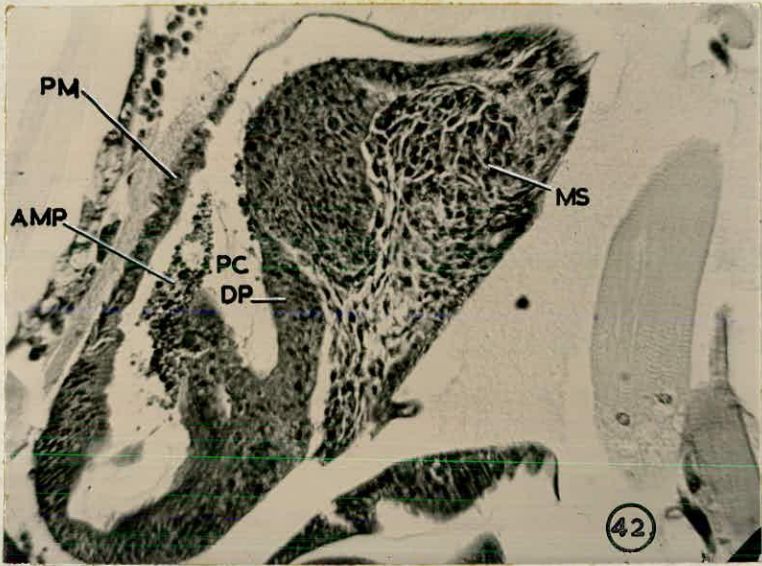


Fig. 43. An oblique section in $5\frac{1}{2}$ day old malignant larva showing physiologic antagonistic action towards metastatised growth. NC - ventral nerve cord; MH - melanised hexagons; SP - spheroids. X 200.

Fig. 44. Clumping of tumour growth around the ventral nerve cord (NC); MC - metastatised tumour tissue; CS - patches of clumped spheroids; SP - spindle cells. X 180

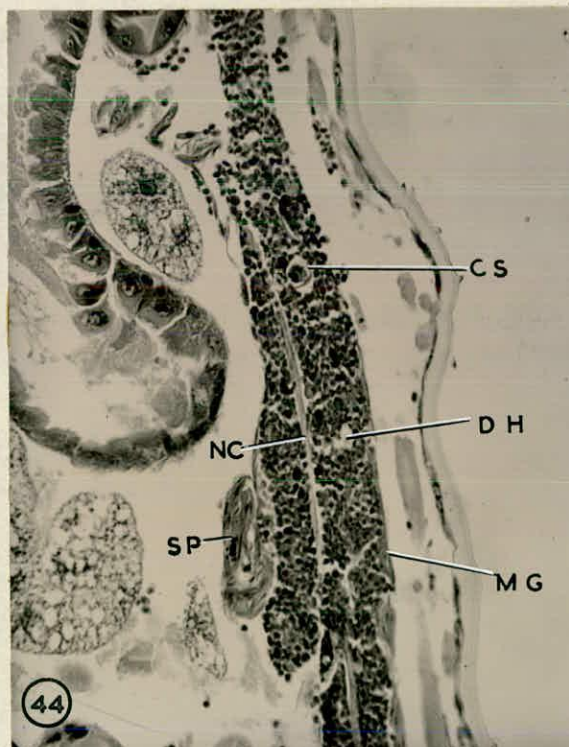
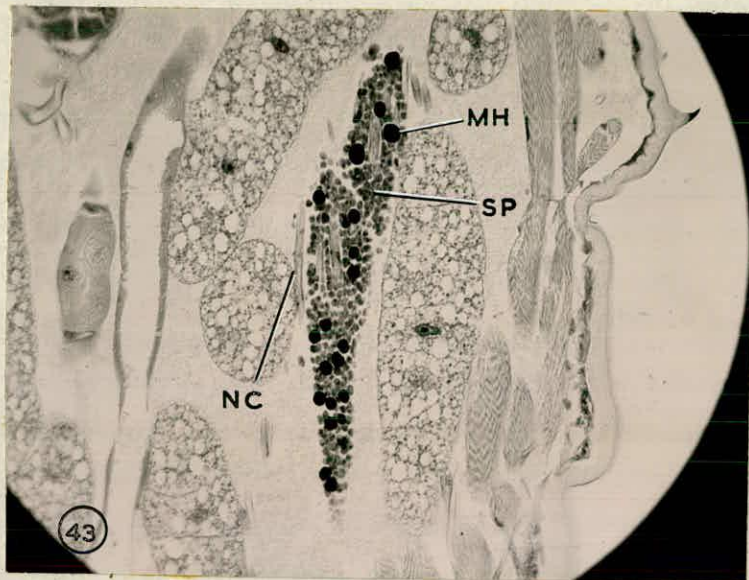


Fig. 45. Neoplasm antagonised after infiltrating the imaginal discs. TC - tumour cells; WD - wing bud; CC - cephalic complex; RG - ring gland; CH - cerebral hemisphere. X 170.

Fig. 46. A binucleated giant capsule spotted from amongst secondary growth. GC - intact binucleated giant cell; ETM - encapsulated tumour cells. X 1000.

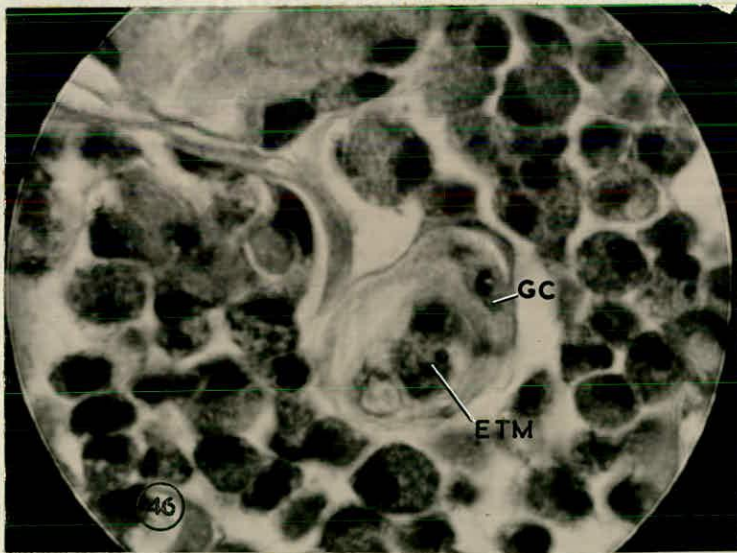
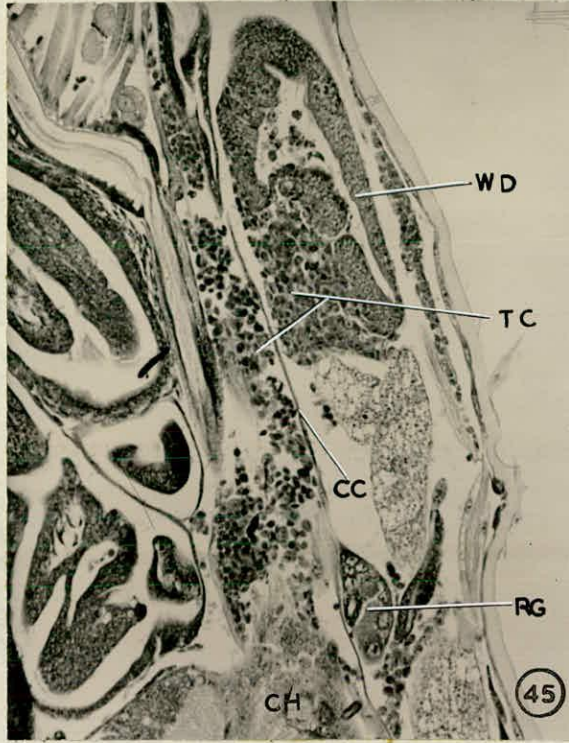
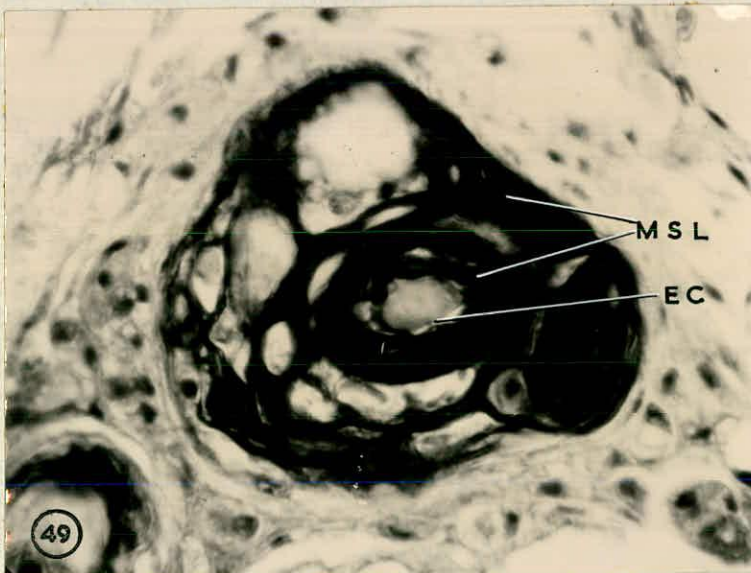
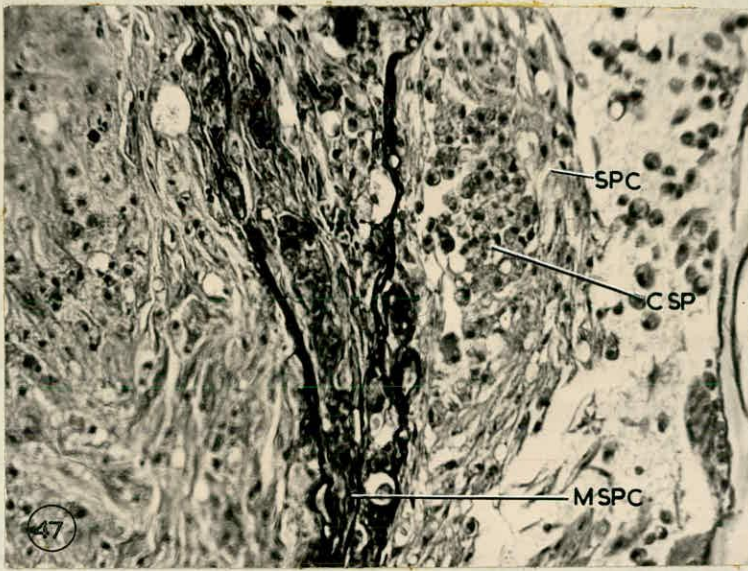


Fig. 47. Formation of the melanised structures from the antagonised tumour cells. SPC - spindle cells; CSP - clumped spheroids; MSPC - melanised spindle cells. X 340.

Fig. 48. Early stages in the disintegration of the clumped spheroids (DSP); MSPL - melanised spindle cells. X 240.

Fig. 49. Early formation of the black bodies. EC - focal substrate; MSL - deeply melanised spindle layers. X 700.



While growth of the black bodies is continuing by the addition of new strata, changes occur in the tumour cells which have already been deposited. The two types of cells behave differently. In the layers of spindle cells there is a deposition of melanin. This occurs only in the walls of the cells, and is accompanied by a gradual disappearance of the nuclei. Meanwhile the enclosed spheroids which have not been transformed into spindle cells never deposit melanin but suffer only action involving lysis. In elder structures lysis and melanin deposition increase in intensity so that all the cells lose their identity (Fig. 48).

It is of interest that these black bodies require a focus around which they are formed. In those formed in the fat bodies, the necrotic tissue serves as the focus and those free in the haemocoel originate when some spindle cells surround any available small structure which may be loose there. Sometimes it is a broken piece of muscle, but in the case illustrated in Fig. 49 the spindle cells surround an unidentified cell which seems to have an intact nucleus and a basal extension and is probably a platelet. It becomes encapsulated by several layers of spindle cells which later become melanised. Such a structure acts as the focus around which the condensation of tumour cells in the manner previously described can take place.

The incidence of black bodies in lethal-malignant larvae is very low. They occur mostly in larvae in which puparium-formation is delayed for four days. The majority of larvae which form puparia earlier do not show them. However, sectioned material of such larvae at stages just before puparium-formation showed that the fundamental processes leading to the appearance of black bodies had already begun to take place, the haemocoel being loaded with aggregates of spheroids and spindle cells.

In these late larval stages the hexagons, unlike the spheroids which transform into spindle cells, become melanised but they soon disintegrate and disappear. They do not normally take part in the formation of the black bodies.

Behaviour of the imaginal discs in larvae in which tumour growth is inhibited early.

In larvae in which, owing to an early inhibitory action of the giant cells, the tumour cells remain confined to the lymph glands and are not released into the haemocoel, there is, of course, no direct contact between tumour cells and the imaginal buds. Nevertheless the latter showed some remarkable abnormalities which are important for later discussion in connection with the somewhat similar conditions found in the lethal-no-differentiation.

The imaginal buds of late third instar wild-type

larvae have all a rather similar general structure. Each is composed, as shown in Fig. 39, of two intimately connected layers; a loose layer of mesoderm cells which is covered by a folded layer of columnar epithelium, the whole being enclosed by a comparatively thin peripodial membrane. The epithelium can be differentiated into a proximal portion which during metamorphosis forms part of the integument of the body and a distal portion which forms an appendage. These two regions are referred to here as A and B respectively. A fuller description of the buds is given at a later stage (pages 49-55).

In the larvae with inhibited tumour growth the mesoderm supply of region B proliferated very rapidly (Fig. 40). This was accompanied by, and was probably a direct cause of, a flattening out of the overlying epithelium which by this stage should already have been folded. We thus found the B portion of the disc consisting of a simple epithelium lying above a rapidly proliferating mass of mesoderm. The appearances strongly suggest that the increasing multiplication of the mesoderm eventually ruptures the epithelium, after which the mesoderm cells escape freely into the peripodial cavity (Figs. 41 and 42). The mesoderm cells up to the stage in the process look fairly normal. In older specimens which seem to have gone through a similar set of changes, the mesoderm cells may undergo

retrogressive changes and break down into a mass of featureless granules. This is accompanied by an increased affinity for stains, possibly due to heteropycnosis.

Mesoderm of region A behaves in a slightly different way. It expands laterally and towards the outer side of the disc but its proliferations appear more normal and less disorderly than that of the mesoderm in region B. It often collects into a large rounded mass (Fig. 42) and causes a thinning of the overlying epithelium (Fig. 40). In later stages, however, the epithelium in A region may become completely ruptured and the mesoderm as abnormal as that of segment B. There is, however, considerable variation in the appearances exhibited by the proliferating mesoderm. The situation is better understood after corresponding abnormalities in lethal-no-differentiation are discussed fully.

Contributions of the lymph gland cells to normal development

The only aspect of the normal development of the lymph gland which has so far been described in this thesis is its histological structure in the mid third instar. The very striking changes that occur in lethal-malignant larvae have drawn attention to the various types of cells comprising the organ and the

transformations which they may undergo. We may now turn to consider some aspects of the developmental performance of these cells in normal wild-type larvae. There are two main periods in normal development at which lymph gland cells are released into the haemocoel. The first of these occurs in the late second instar, the second in the late third instar. Here we shall discuss only the first of these. The second will be dealt with later in the light of information required in the study of another mutant, lethal-benign (page 69).

The lymph gland cells released at the end of the second instar are studied firstly in relation to the imaginal discs, and secondly to the ventral hypodermal organ which itself is connected with the circulatory system. We shall also have to discuss the possibility, favoured by certain earlier workers, that the released lymph gland cells can be regarded as blood cells or leucocytes.

Imaginal primordia

In late larval stages of Drosophila, or in Diptera in general, two different kinds of tissues could be distinguished - imaginal and larval. The former are made over to the imago during pre-pupal and pupal stages. In the meantime most of the latter break down and entirely lose their identity. But some of them remain essentially intact, showing no morphological

alterations, and form some organs of the adult, e.g. the heart and Malpighian tubules (Strasburger, 1932; Robertson, 1936, and others).

The two events of construction and destruction happen simultaneously and comprise the most remarkable changes in the development of Diptera. They are grouped together as metamorphosis. The two differently potential tissues differ in their affinity towards basic dyes. Imaginal primordia, as compared with larval tissues, stain deeply with haematoxylin. This is probably due to their high content of nucleic acids. Imaginal tissues increase by cell division, while the purely larval ones grow only by increase in cell size.

The following description of the imaginal anlage is based on late third instar control material. By far the most important group of imaginal primordia, which give rise to most of the adult structures, comprise a group of imaginal discs or buds lying in the thoracic region of the larvae. The majority of the discs fall into two groups - a lateral and a ventral set. The ventral one includes two pairs of buds; ventral prothoracic (fore-leg buds) and ventral mesothoracic (second pair of leg buds). The lateral set comprises three pairs; dorsal mesothoracic (wing discs), dorsal metathoracic (halter discs) and the ventral metathoracic (third pair of leg discs). There is also an unpaired disc lying ventrally in the last abdominal segment. This is

the genital disc, which metamorphoses to genitalia and connected elements. In normal conditions, imaginal discs contribute not only to the imaginal appendages (wings, legs, halteres, etc.) but, in addition, the cephalon and thorax are derived from particular parts of them. In each imaginal disc of either the ventral or lateral series two parts may be distinguished - a folded distal portion and a plane proximal one. In metamorphosis, they give rise to the limb proper and corresponding parts of the thorax respectively. The imaginal thoracic hypodermis is composed of a dorsal tergum and a ventral sternum. Hence, the proximal parts of the dorsal pairs of discs metamorphose to the thoracic dorsal integument; those of the ventral set develop in turn to the thoracic sternum. While the former portions grow latero-dorsal to fuse in the mid-dorsal line, the latter fuse in the mid-ventral line, so that a complete imaginal thorax is finally formed.

The eye-antenna complex and pharyngeal buds complete the series of imaginal discs. The former compound structure is represented by a pair of discs lying medio-dorsal and in direct connection with the brain through optic nerves. It enters into the formation of the eye and antenna with their accessory structures and of the cephalic hypodermis. The pharyngeal bud forms the imaginal pharynx and associated muscles.



Early post-embryonic development of imaginal discs

Several workers have analysed the post-embryonic development of Drosophila, paying particular attention to larval stages in which differentiation of the imaginal primordia begins. The following description of embryonic imaginal buds is compiled mainly from Auerbach (1936). Newly hatched larvae show the anlagen of all the ventral buds as small thickenings of the hypodermis. During the first and the greater part of the second instar the differentiation of the leg buds proceeds no further. The buds continue to form parts of the hypodermis, having the form of posterior solid outgrowths directed towards the posterior. Towards the end of the second instar they invaginate into the interior of the larval body cavity, but continue to connect with the body wall by a stalk. Invagination results in the appearance of the peripodial cavity, which is surrounded by the invaginated hypodermis. The latter has indications of a differentiation involving a slight dorsal thickening, brought about by the elongation of the cells. This thickened part later forms the disc proper, while the remaining portion is the peripodial membrane surrounding the peripodial cavity into which the anlage of the leg later protrudes.

According to Auerbach on Calliphora (1936), the wing and halter buds (lateral set) are found in newly hatched larvae at a more advanced stage than the ventral

buds. By that time, they have already invaginated and constricted off from the hypodermis as small sacs with a distinct lumen. The same situation, as verified by the same author, prevails in Drosophila, where the buds were found to be already invaginated to the same degree as in Calliphora.

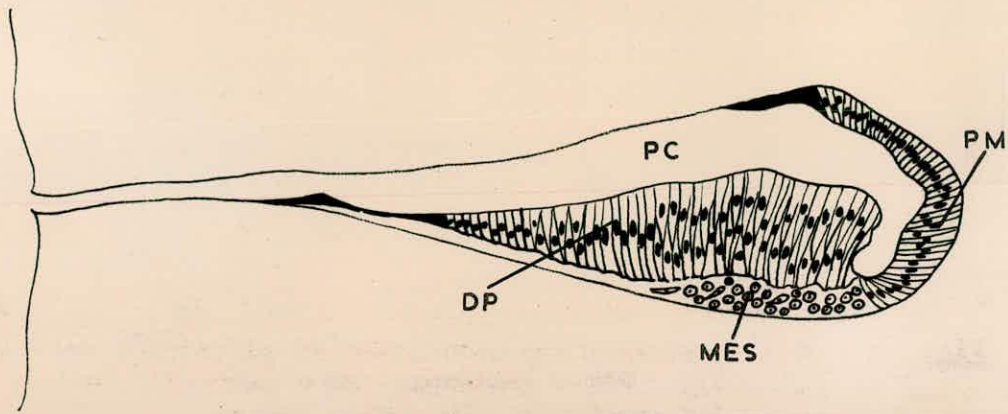
Imaginal disc mesoderm.

By the end of the second instar, a new structure, the mesoderm, establishes itself under the thickened portions of the imaginal discs. Thus a section in an imaginal disc, as illustrated in Fig. 50, shows the disc proper as a simple, single-layered, thick epithelium bordering on the peripodial cavity and overlying a loose tissue of mesoderm cells. The origin of the mesoderm has been much discussed, but the processes leading to its sudden appearance have remained obscure. However the present investigation of the behaviour and post-embryonic development of the blood-forming organs has yielded results related to its mode of formation and these are discussed below.

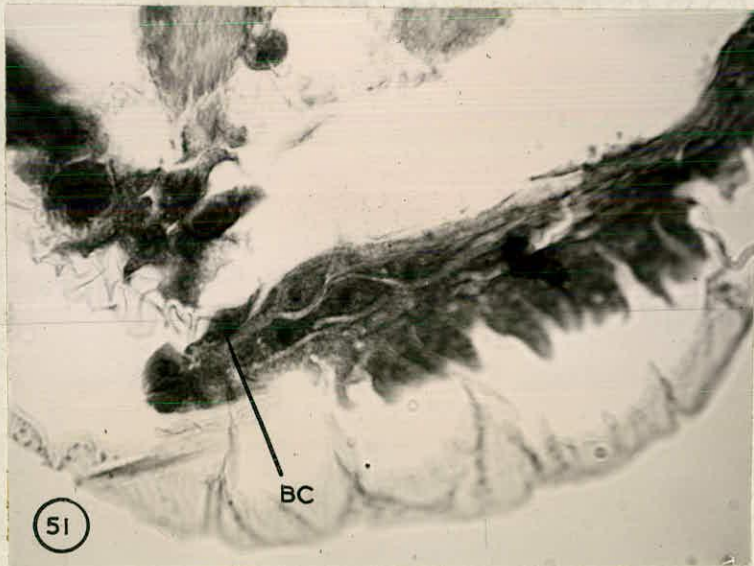
At the end of the second instar, the first pair of lymph glands releases some of its cells, comprising both spheroids and hexagons. The former move forward to the region where the young imaginal discs occur. They accumulate underneath the disc proper and constitute a compact tissue of loose cells. These groups of cells, with which every imaginal disc is provided, are the

Fig. 50. A diagrammatic representation of an early third instar imaginal disc. MES - mesoderm; PC - peripodial cavity; PM - peripodial membrane; DP - disc proper.

Fig. 51. A section in the posterior part of a late second instar wild type larva showing the mode of formation of the ventral hypodermal organ (lymph hearts) from migrating lymph gland cells. BC - boat-like cells. X 800.



50



51

mesoderm supplies. It has not yet been possible to give an experimental demonstration of this, since, as will be shown later (page 12), the lymph glands behave in a peculiar way when one attempts to transplant them from one larva to another. However, the evidence that the lymph glands release cells at exactly the time when mesoderm is appearing in the imaginal buds and the precise histological similarity between the two groups of cells, leaves no doubt that it is from the lymph glands that the imaginal disc mesoderm takes its origin.

As described by Auerbach (1936) and other workers, the imaginal disc proper invaginates into the peripodial cavity which is thus reduced in size. This invagination leads to the appearance of another cavity lying beneath the disc proper, which receives the migrating mesoderm. While it is invaginating, the thick epithelium of the disc begins to be folded. Waddington (1942a and b) has argued that this process of folding plays an essential part in the determination of the type of imaginal differentiation which the bud will perform. The question arises as to the relative roles played by the epithelium and by the mesoderm in producing the folds. Histological examination of the process very strongly suggests that it is the mesoderm which is the primary factor in this process. We have already seen that in certain individuals of lethal-malignant in which the growth of the tumour tissue is inhibited at an

early stage by the antagonistic processes which occur, the mesoderm of the imaginal buds may yet become abnormal and that this seems to have secondary consequences on the overlying imaginal epithelium. We shall see later (page 61) that the same type of phenomenon is better exhibited in lethal-no-differentiation. The appearances, in fact, suggest that the migrating mesoderm cells by their proliferation exert a mechanical pressure on the overlying epithelium causing it to bulge into the peripodial cavity. It is, of course, difficult to prove that nothing but a mechanical pressure is involved in this, but we shall see later a whole range of phenomena which seem to be adequately explicable in terms of that simple hypothesis and it may therefore be retained until it becomes necessary to introduce more complex ideas.

The migration of presumptive mesoderm cells from the lymph glands continues rapidly at the end of the second instar until a good number of them has established itself in every imaginal disc. The cells undergo some slight morphological modification, becoming oblong rather than spheroidal. However, even in later stages of the discs the majority of the cells have not altered very much from their original form.

The ventral hypodermal organ and the circulatory system.

In late larval stages of Drosophila the ventral hypodermis of the last abdominal segment appears different from the rest of the hypodermis. Morphologically it acquires the form of a paired organ distinguished by its convex curvature and by being elevated from the rest of the body. Microscopically it is several times as thick as the rest of the hypodermis, and also differs from it by being syncytial; that is to say, it is not divided into cells but the nuclei are uniformly scattered in a continuous mass of cytoplasm. This organ is macroscopically visible at the end of the second instar, and becomes quite voluminous by the end of the third. It has not attracted attention in Drosophila literature and probably has not been reported in other Diptera.

Formation

At the end of the second instar, some of the freed spheroids crowd into the haemocoel of the last abdominal segment, where they change from a rounded to a boat-like shape. They penetrate the ventral hypodermis of that segment and arrange themselves perpendicularly on it (Fig. 51). This is accompanied by the secretion of a large amount of cuticle. When this preliminary configuration is attained, more cells, after acquiring the boat shape, rest over the original layer with their cell tips touching. This process is

repeated rapidly until the hypodermis in that region becomes multi-stratified. Each layer is a row of cells which connect laterally with each other and alternate with the cells of the surrounding layers. Later the organ loses its cellularity and transfers to a syncytium. The relation of this organ to the circulation is dealt with, following a short summary of the circulation in Diptera.

The circulatory system.

The circulatory system in Drosophila larvae, like that of the adult, and of other Diptera, is largely an open one, there being only a single closed organ or dorsal vessel. The greater part of the circulation of the blood takes place in the cavities of the body; the blood occupying the spaces not appropriated by the internal organs. These spaces are called sinuses, and are separated from each other by septa or diaphragms. When the diaphragms are completely developed the general body cavity or haemocoel is divided into three sinuses by means of two fibromuscular septa. The dorsal diaphragm extends across the abdominal cavity above the alimentary canal and the blood space thus enclosed is known as the dorsal or pericardial sinus, within which the heart is located. Pairs of alary muscles, composed of striated fibres, arise from the terga and spread out fanwise over the surface of the dorsal diaphragm. The fibres of the

alary muscle of one side meet those of the corresponding muscles of the opposite side of the body beneath the heart. The ventral diaphragm stretches across the abdominal cavity above the ventral nerve cord, and the space limited by it is the perineural sinus. The space between the dorsal and ventral diaphragms is the main body cavity and constitutes the third or visceral sinus.

The heart is the principal pulsatory organ and it undergoes rhythmical contractions which are brought about by the muscle fibrillae situated in its walls. The colourless blood enters from the pericardium to the heart through ostia guarded by valves, so that when the heart contracts the blood is forced forward only. The alary muscles control the flow of blood into the pericardium and hence by alternate contraction of the alary muscles and the walls of the heart the circulation is maintained. In insects, however, accessory pulsatory organs have been described. A technique has been developed by the writer to recognise organs of pulsatory nature in Drosophila larvae. This depends on superficial examination under a low power binocular of etherised larvae stretched and fixed on a slide. This treatment affords excellent material for following the direction of the circulating haemolymph through the transparent body wall. By this method it can be seen that the mid gut plays an

important role in directing the haemolymph of the visceral sinus. Their rhythmical contraction both backward and lateral, sucks in the haemolymph pouring anteriorly from the dorsal aorta to bathe the visceral organs and hence maintains a continuous circulation from and to the dorsal sinus. However the anterior loose masses of fat bodies and Malpighian tubules are also pulsatory, and affect the circulation in a similar way to the mid gut.

It was through this technique that a function was attributed to the paired ventral hypodermal organs of the last abdominal segment. They were found to undergo rhythmical contraction, probably corresponding to that of the heart, and therefore function as important pulsatory organs. The posterior part of the body cavity is practically devoid of viscera which might act as pulsatory tissues and represents a relatively big space for the accumulation of the haemolymph. The occurrence of this organ in this region is therefore significant, as it directs the body fluid dorsally in the direction of the pericardial sinus and so prevents the stagnation of haemolymph. Thus, in function, the ventral hypodermal organs correspond to vertebrate lymph hearts and this name may be given to them.

Blood cells

Extra lymph gland cells which remain after the formation of the mesoderm, lymph hearts and several

other structures, collect in the last abdominal segment and adhere to its hypodermis. They are currently accepted as colourless blood cells or leucocytes. Balbiani (1886) was the first to attribute to the lymph glands the formation of blood cells. Cuenot (1891) considered this structure similar to the lymph glands of vertebrates and capable of giving rise to blood cells and phagocytes. Kollman (1908) and Stark and Marshall (1928), after an extensive review of the entire problem, came to the conclusion that the lymph glands are, in fact, a lymphoid tissue serving as an organ for the replacement of blood cells and phagocytes. Investigations of a large amount of sectioned material carried out by the present worker failed completely to substantiate the validity of these findings. The lymph glands form the tissues (imaginal mesoderm, ventral hypodermal organs) which have been described, and, as explained later, are probably endocrine organs with a secretion affecting the differentiation of some of the imaginal primordia. But there has been no supporting evidence that these cells, usually assumed to be blood cells, are really so. It appears that the haemolymph of Drosophila larvae is devoid of haemocytes comparable with those of other animals. These alleged blood cells remain confined to the hypodermis of the last abdominal segment from the time they emerge at the end of the second instar till the third instar

is completed. None of them was ever found circulating in the blood stream. That is to say, that sections reveal a transparent body cavity. Moreover, serial sections through the whole of the dorsal vessel showed no trace of them. At about the middle of the third instar, these cells undergo structural distortion, partially in the form of nuclear breakdown, so that their relation to the circulation or any other system is most improbable.

This conclusion is supported by unpublished work on "lethal no-discs" where blood-forming organs are wanting. This is accompanied by the entire absence of imaginal buds and of the so-called blood cells. Yet the larvae are as big and healthy as control; and lethality is only due to their failure to form prepupae.

Lethal-no-differentiation

As the name implies, the effect of this lethal is to inhibit differentiation of the imaginal discs into the appendages and body segments of the adult. The inhibition sometimes affects all discs and sometimes only some of them. As we shall see, there are interesting regularities in the patterns of discs which are affected.

The lethal larvae appear normal, but puparium formation is retarded for a variable period up to a maximum of two days. The lethal individuals usually die as pre-pupae or early pupae. After death they

disintegrate so that the old dead specimens appear as empty hard puparia.

The internal structure of first and second instar larvae seems quite normal. Deviations from the wild type begin to be noticeable within the period from about 15 to 30 hours after the beginning of the third instar. The first abnormalities to be visible are defects in the lymph glands. The pathological picture of these is shown in Fig. 52 which was taken from a 25 hour old third instar larva. As shown, the hexagons have become deeply melanised, and the spheroids clumped together forming patches or groups of distorted cells. Later stages, up to the end of the third instar, showed both types of cells completely histolysed with the result that melanin is dispersed in the stroma. However, some hexagons disintegrate completely, and rounded vacuoles representing their original site are of frequent occurrence. Spheroids too gradually disappear and add to the stroma. Old defective glands show no definite organisation. This is easily demonstrated in late third instar material.

Studies on earlier lethal lymph glands demonstrated that, while hexagons show a faint degree of melanisation marking the onset of their degeneration, the spheroids are at least morphologically normal. This condition lasts for a short period after which the latter also exhibit abnormal peculiarities. The fate of platelet

cells was exceedingly difficult to follow and was therefore left for more detailed analysis.

Imaginal discs

Soon after the lymph glands become abnormal, pronounced pathological changes occur in the imaginal discs. At an early stage of lethal action, the imaginal disc mesoderm, which is represented by bands of loose cells underlying the disc epithelia, begins to proliferate rapidly and different patterns of damage are produced, as follows:

- 1) Mesoderm multiplies rapidly in the direction of the overlying disc epithelium, which is represented by a single layer of columnar epithelial cells (Fig. 53). The resulting cells, however, are phenotypically identical with those of the normal dividing mesoderm. Vigorous cell multiplication continues till the tissue becomes several times as voluminous as in wild-type. Eventually it penetrates the corresponding disc proper, causing it to rupture, and the rapidly growing mesoderm reaches the peripodial cavity, which affords more space for its expansion. In the peripodial cavity cell proliferation proceeds in rather unique ways, one of which is illustrated in the fig., which shows that the mesoderm acquired the form of a stalk bearing a mushroom-like cap which has grown in all directions to occupy most of the available space of the peripodial cavity. Later the mesoderm as a whole undergoes marked destruction, exhibited in loss of cell identity

and nuclear breakdown.

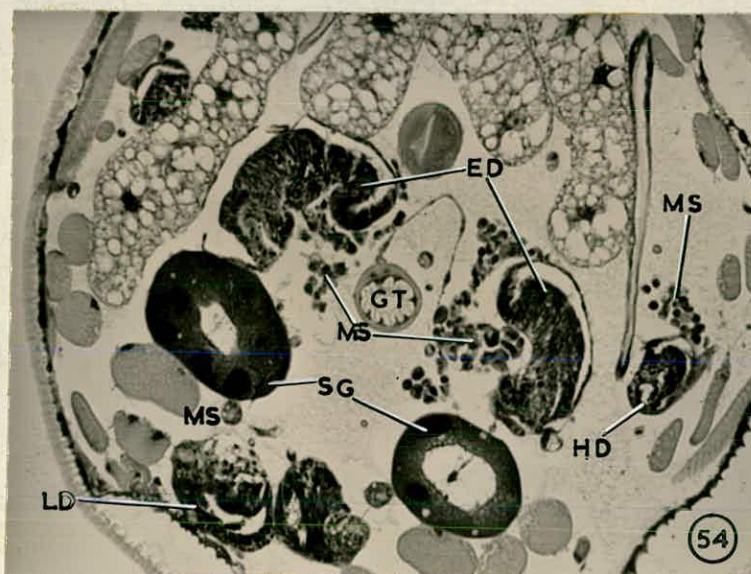
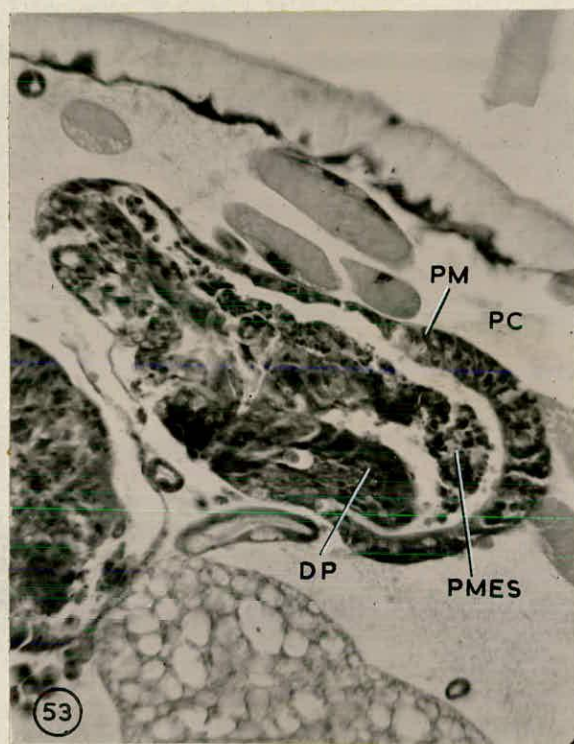
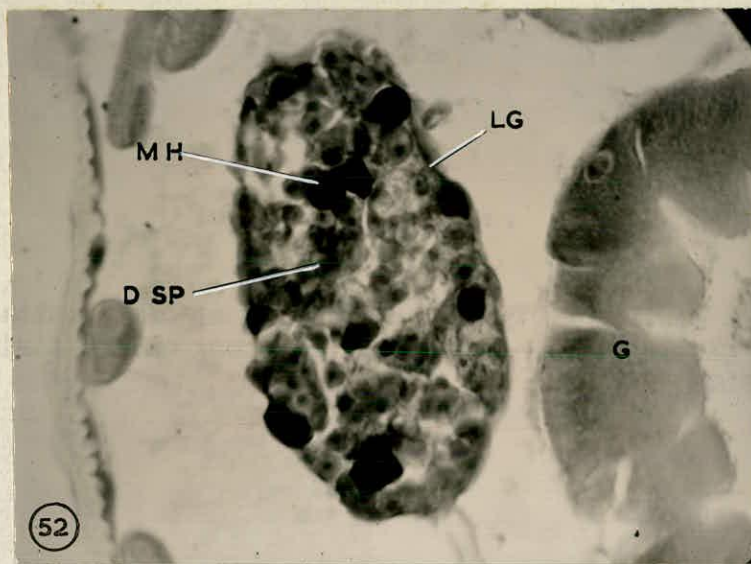
- 2) Another pattern of damage is shown in Fig. 54, where the mesoderm multiplied in the direction of the body cavity. This results in a continuous flow of cells pouring into the haemocoel. Consequently the disc epithelia remain unaffected.
- 3) A third configuration showed the mesoderm had multiplied uniformly, lifting up the overlying disc epithelium without penetrating it. Active division was accompanied by more uplift until the epithelium was forced by the overwhelming pressure underneath into close contact with the peripodial membrane at the other surface of the imaginal disc. In such cases the peripodial cavity is obliterated.
- 4) The last type so far examined shows milder consequences. The mesoderm undergoes a considerable amount of increase, sufficient to unfold the already segmented disc proper. However, an antagonising mechanism seems to have checked the growth of the mesoderm, which appeared more basophilic than usual, and degenerates at an early stage.

The affected imaginal discs could quite as easily be studied in whole mounted material. Discs from fixed larvae are dissected out by a tungsten needle and stained with Delafield's haematoxylin without differentiation in alkaline solutions. Fig. 55(a) is a surface view of a whole mounted wing disc showing

Fig. 52. A section in 75 hour old lethal no-differentiation lymph glands. MH - melanised hexagons; DSP - disintegrating spheroids. X 600.

Fig. 53. Section in a 15 hour ^{3rd instar} old lethal no-differentiation larva showing behaviour of the imaginal discs. DP - disc proper; PM - peripodial membrane; PMES - proliferated mesoderm cells falling in the peripodial cavity (PC). X 400.

Fig. 54. ^{3rd instar} Transverse section in the anterior region of a 25 hour old lethal no-differentiation larva. MS - mesoderm proliferating in the haemocoel; ED - eye disc; GT - gut; SG - salivary glands; HD - haltere disc; LD - leg disc. X 230.

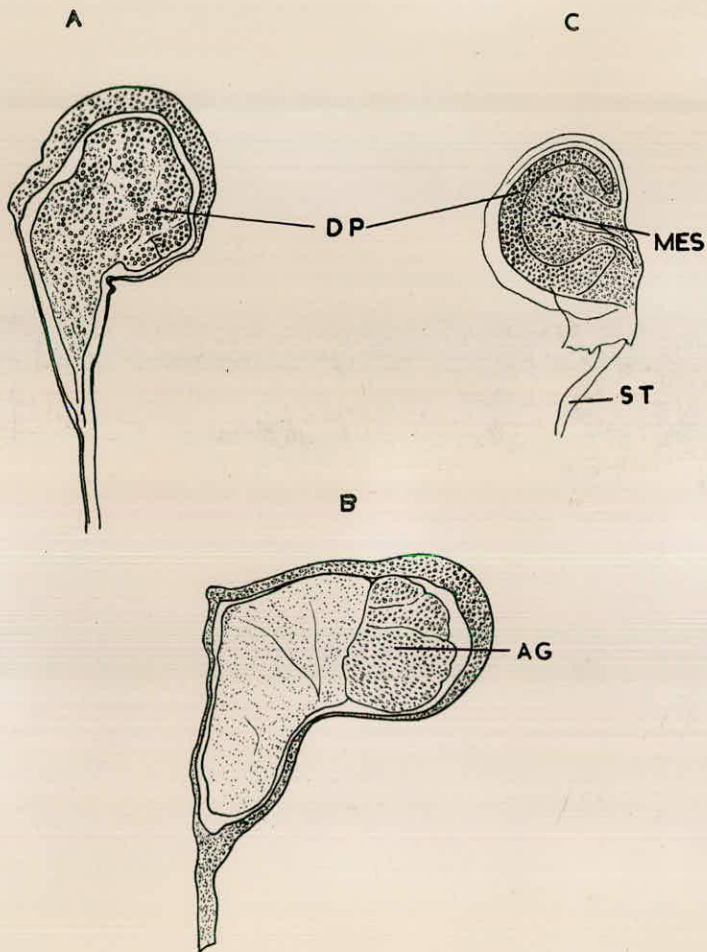


the absence of the characteristic subdivision of the corresponding normal organs. Fig. 55(b) is also a wing bud from another specimen, again showing unsegmented epithelium. In addition the mesoderm at the proximal end of the disc has penetrated the disc proper in that area. Fig. 55(c) is a lateral view of a haltere bud in which the mesoderm has become much hypertrophied in size, and the disc remains undifferentiated.

In the course of metamorphosis, imaginal discs, as mentioned earlier, give rise to imaginal limbs and other adult structures. The present studies suggest that the eversion of discs at the beginning of prepupal life is mechanically induced by forces resulting from the proliferation of the mesoderm in particular directions. Furthermore, the mesoderm itself metamorphoses to muscle cells of the limbs, thorax and head, while disc proper forms limb epithelia and other organ integument. As a result of the defects of the imaginal discs which date back to larval life the imaginal structures they produce must be expected to be abnormal. These abnormalities will be caused partly by the failure of the discs to evert owing to the degeneration of the mesoderm and partly as a consequence of the rupture of the disc epithelia.

Some lethal individuals die as late third instar larvae or early pre-pupae. Those which succeed in developing to the pupal stage present a most interesting

Fig. 55. Whole mounts of defective imaginal discs (lethal no-differentiation). a and b represent abnormal wing buds while c is a haltere bud. DP - disc proper; MES - mesoderm; ST - stalk; AG - region of disturbed mesoderm. Camera lucida.



distribution of abnormalities which may be summarised as follows:-

- 1) In some (Fig. 56) only the legs are markedly abnormal. It is always found that all three pairs of legs, despite their basically different origin from different imaginal discs, are always abnormal to about the same extent and exhibit very similar patterns of damage. The same phenomenon has been found in work on other lethal stocks with limb abnormalities in which again the three pairs of legs are usually similarly affected.
- 2) Individuals with defective eyes, as shown in Figs. 57 a and b (which represent ventral and dorsal views of the same specimen) always have abnormal legs.
- 3) Wing abnormalities (Fig. 58) are always accompanied by both leg and eye defects.
- 4) A considerable number of lethal individuals was found in which the thorax was absent (Fig. 59). These always had wing, eye and leg abnormalities in addition.
- 5) In pupae showing the most extreme conditions the genital disc failed to differentiate. These specimens always showed an absence of the thorax as well as wing, eye and leg defects.

These facts show that the abnormalities are not distributed haphazardly but are connected in an orderly

manner, the weakest grades of defect impinging only on the legs while more severe defects involve successively the eyes, wings, thorax and the genital system. This sequence, which is visible in the pupae, can also be found in the histological examination of the larval imaginal discs. Larvae in which the eye discs were affected always showed disturbed leg discs; those with abnormal genital discs have all their imaginal discs distorted, and so on.

The most common eye defect involved the unilateral bulging of an eye or the duplication of eyes. In the specimen shown in Fig. 57, two eyes exhibit very different features. The right one is represented by an elevated, irregular outgrowth in the shape of a pointed lobe which has shifted from normal position to bend backwards towards the thorax. This can be seen in the ventral view in Fig. 57a. It is composed of two unequal parts separated from each other by an ill-defined suture. The left eye is roughly rectangular instead of round, as is the wild-type eye. Neither eye showed differentiation of ommatidia or pigment deposition. Although in some cases the two duplicated eyes on one side were together equal in size to a normal eye, in others there was one eye as large as normal accompanied anteriorly by a smaller one.

In specimens in which the wings were affected, the abnormal wings were always very similar to one another.

Fig. 56. Ventral view of a whole mounted lethal pupa. LG - abortive wing; THO - thorax; WG - wing; AD - abdomen; PM - pupal membrane. Camera lucida.

Fig. 57. Ventral and dorsal view of the same lethal specimen showing abnormal eyes and legs. AE - protruded eye; BE - bilobed eye; MP - mouth parts; TH - thorax; LG - legs; AD - abdomen; PM - pupal membrane; WG - wings. Camera lucida.

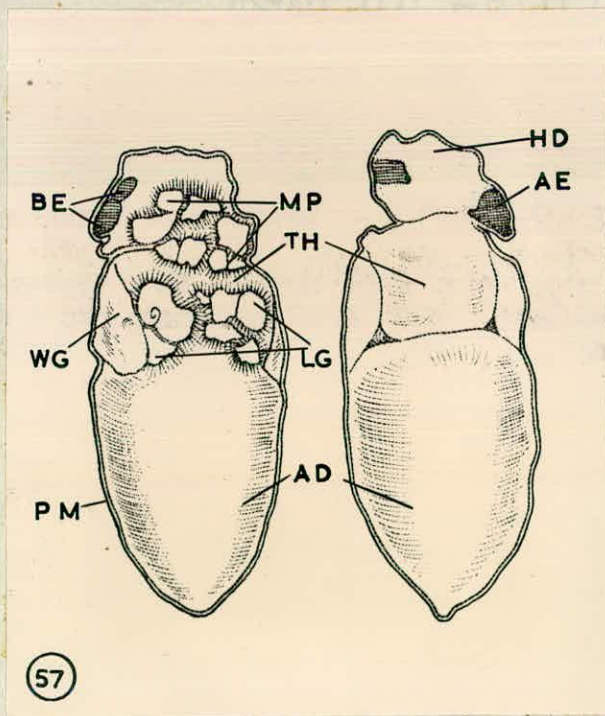
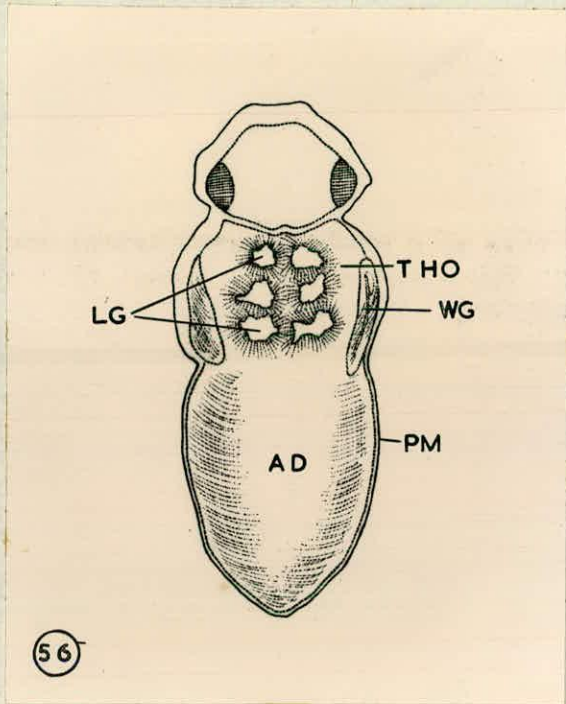
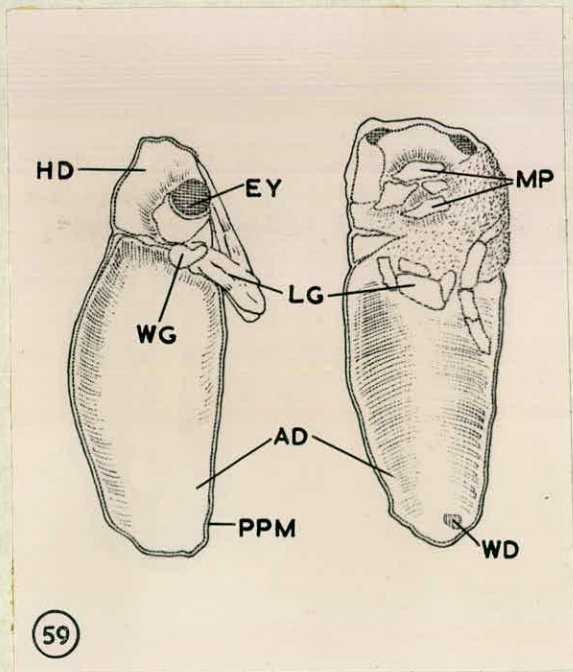
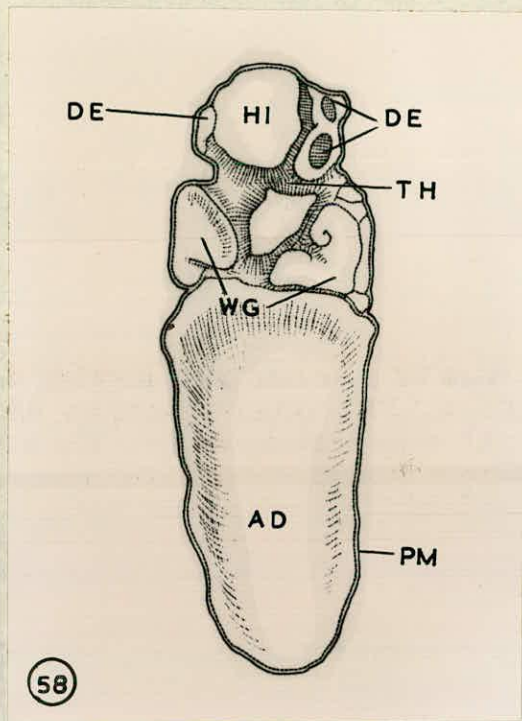


Fig. 58. Dorsal view of a lethal pupa showing defective eyes (DE); WG - wings; PM - pupal membrane; AD - abdomen; TH - thorax; HI - head integument. The abnormal legs are not seen. Camera lucida.

Fig. 59. A lateral and ventral view of a no-thorax pupa showing defective eyes (EY), wings (WG), legs (LG) and mouth parts (MP). AD - abdomen; WD - genital disc; HD - head.



They consisted of thick lumps of undifferentiated tissue not properly articulated to the thorax. Normally the wings grow laterally and ventrally to cover the ventral side of the thorax and much of the abdomen. Lethal wings, as shown in Fig. 58, tend to be turned dorsally to extend over the thoracic terga and they never reached the abdomen.

The individuals lacking the thorax always formed dwarf pupae, their small size being due not only to the entire absence of the thorax but also to a remarkable reduction in the size of the head and abdomen. The wings in these specimens are represented only by very small lateral protrusions which project from between the head and the abdomen. The legs often show some signs of segmentation but they are for the most part concealed by a nondescript tissue which represents the histolysed larval organs in that region. The external genitalia, however, may look normal, which implies that the genital disc has developed normally.

The affected legs generally appear as unsegmented growths connected with the thorax. They never extend posteriorly so as to cover the abdomen, as happens in wild-type pupae. Sometimes they elongate, giving rise to structures with no very definite pattern, but even these are crowded on to the ventral surface of the thorax. A few cases were found in which the abnormal

legs showed the preliminary stages of segmentation. Usually, however, the stage of development did not exceed that illustrated in Fig. 56, where the legs appear as lobular patches of tissue.

Measurements of various parts of the internal organs which are not formed from the imaginal buds, such as regions of the alimentary canal, the testes, salivary glands, etc., showed no difference between the lethal larvae and their wild-type counterparts. Similar measurements show that the imaginal discs, on the other hand, besides exhibiting the histological abnormalities already described, are somewhat smaller than normal in their overall dimensions.

It is possible that the lethal phenotype involves other abnormalities than those of the lymph glands and imaginal discs, but these have not yet been detected.

Lethal-Benign

This stock was selected for study partly in connection with certain investigations not connected with the present study, but also to provide material for the examination of the melanised black bodies, which are the entities usually referred to when Drosophila tumours are discussed. The similarity in structure in these bodies in the normal strains of benign tumours, which have been studied in several different laboratories, and in the black bodies found

in the malignant lethal larvae suggests that they originate by similar processes of histogenesis. Since in the malignant larvae the black bodies are the end-result of a long process which starts with abnormalities in the lymph glands, it seemed particularly important to inquire into the behaviour of these glands in strains showing benign tumours. Since in the benign tumour races these bodies usually appear earlier than they do in the malignant race, it seemed possible that lymph gland changes would also be detectable at an earlier stage.

In the strain of lethal-benign selected for study, the first and second instar larvae are normal both in their gross morphology and in their histological anatomy. By the mid-third instar the larvae deviate phenotypically from wild-type by being slightly smaller in size. Towards the end of the same instar, free-moving black bodies are easily visible in all the lethal larvae. They are mainly confined to the caudal end of the haemocoel. The number in which they occur is probably not of any great importance, since in some individuals there may be only one large one, while in others there may be several smaller ones. Shortly after the appearance of the black bodies the larvae stretch out on the walls of the culture dishes and die for causes which are still unexplained. Studies which have so far been made on this stock are

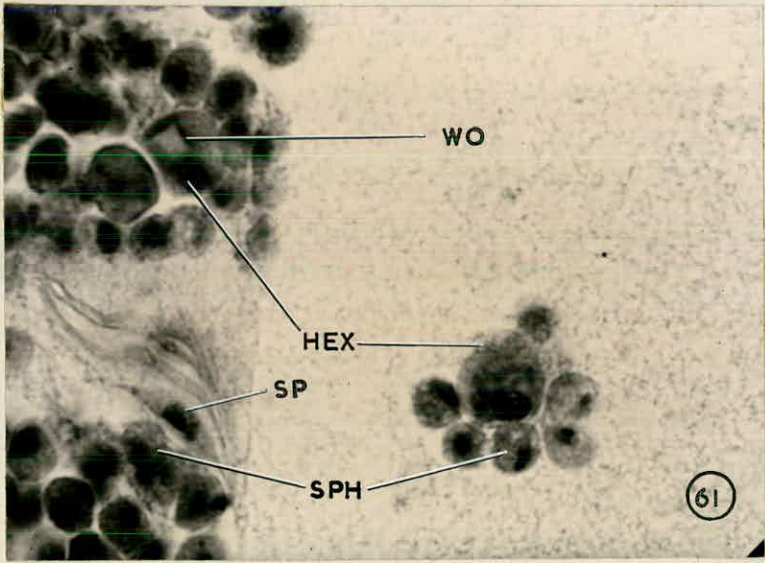
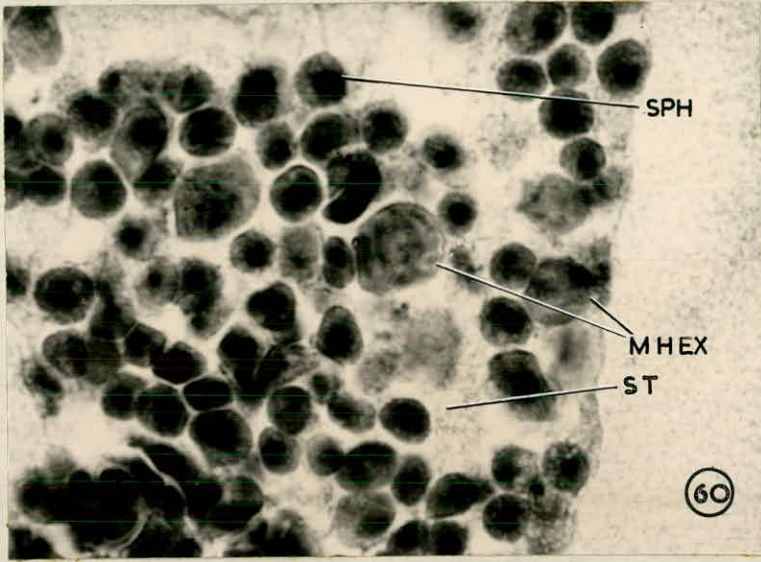
confined to the investigation of the tumours and the actual cause of death is still under investigation (but see page 84).

Blood-forming organs

75-hour old larvae show normal glands and the other organs are also normal. About 5 hours later, the hexagons show a slight degree of melanisation accompanied by morphological distortion, the cells becoming wrinkled and no longer retaining their original hexagonal outline. Fig. 60 shows 4 such cells while Fig. 61 shows another view of a slightly melanised hexagon with a more or less intact upper nucleus below which a window-like opening has appeared, probably in the position of the lateral nucleus. Whether it is a crystal or an extirpated rectangular part of the cell has not yet been decided. 85-hour old larvae show the stroma liquidated and the gland outer epithelium ruptured in several places. Spheroids, which are of normal appearance, have already poured out into the haemocoel. The hexagons, which by this time are dead, are also thrown out from their original position. This is accompanied by a curious occurrence which is illustrated in the right half of Fig. 61 where the spheroids arrange themselves in a continuous ring enveloping a dead hexagon. Such structures were frequent. Approximately at 87 hours, spheroids both in the lymph glands and haemocoel begin to proliferate

Fig. 60. Section in the blood-forming organs of 85 hour old lethal benign larva showing melanised hexagons (MHEX).
SPH - spheroids; ST - stroma.

Fig. 61. Section in the lymph gland region (lethal benign).
WO - window opening below the top nucleus of a binucleated hexagon (HEX). SP - spindle cells; SPH - spheroids. X 300.



rapidly. The lymph glands eventually become much larger than normal and the body cavity is filled with the liberated spheroids. However, nuclear breakdown and spindle cell formation follow. The cells exhibit a disintegration of their big hyperchromatic nuclei, the majority of which break down to a variable number of granules. A large number of spheroids transform to spindle cells, identical with those found in control and in the early formation of the melanised structures already described in lethal-malignant. Fig. 61 shows some of these cells within the blood-forming organs.

Processes of formation of the black bodies in the lethal-benign larvae are essentially the same as those in malignant larvae, the only difference being that they are always formed free in the body cavity. For the formation of these structures in malignant larvae, focal substrates are required. In the many preparations of lethal-benign investigated, however, such foci were not found. This may be held to be a slight histological difference between the structures in the two stocks.

If in an etherised but living larva pressure is applied with a dissecting needle to the neighbourhood of a black body, this can be easily moved around within the organism if it is floating freely in the haemocoel, whereas if it is formed following secondary malignant attack on the fat bodies it remains anchored in place. This rapid method can be applied to large numbers of

larvae and allows one to conclude that truly malignant growths do not occur in the lethal-benign stock.

Histological examination also shows that neither the fat bodies nor the imaginal discs are invaded by malignant cells. However, after the lymph glands exhibit their pattern of damage, the imaginal discs show abnormalities similar to those described in lethal-no-differentiation. There is a proliferation of the mesoderm accompanied by or causing an unfolding of the overlying epithelium. However, since the larvae do not live long after the lymph glands become abnormal, only a few cases were found in which actual rupture of the epithelium had occurred.

The most striking feature of the lethal phenotype, other than the accounts of the black bodies, is the occlusion of the mid-intestine, the lumen of which becomes completely obliterated. This is illustrated in Figs. 62 and 63, which show the features of the ventriculus and stomach and the rest of the mid-gut respectively. In the wild-type the mid-intestine of the mature larva has an inner layer of large epithelial cells bounded by an external basement membrane. Between the epithelial cells and just under the basement membrane are scattered groups of so-called "small imaginal cells" which according to Strasburger (1932) and Robertson (1936) and others are merestimatic and proliferate at the beginning of prepupal life to

give rise to the imaginal mid-gut epithelium. These are distinguished from the epithelial cells both by their staining capacity and their general morphology. Investigation of the lethal-benign larvae showed that the occlusion of the alimentary canal is brought about by the larval mid-gut epithelial cells and not by the alleged imaginal primordia which, contrary to their assumed meristematic nature, do not exhibit any cell-dividing activity. In most cases, however, they persist as undeployed groups of cells. At the beginning of the process of occlusion the larval mid-gut cells enlarge considerably and their free ends become cut off and fall into the mid-gut lumen. This process continues until the lumen is heavily loaded with such cells, some of which are nucleated while others lack a nucleus. Later these proliferated cells disintegrate and are represented eventually by a homogeneous mass of undifferentiated substance filling up the whole of the mid-gut cavity. It is important to note that this process of occlusion of the alimentary canal takes place at about 85 hours after egg-laying when the lymph glands already exhibit their characteristic pattern of damage.

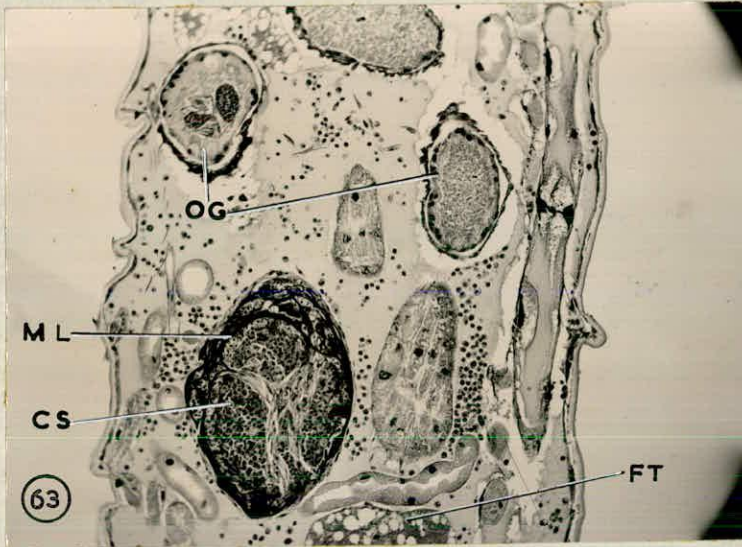
The final stages in the development of the wild-type lymph glands.

It was mentioned earlier that the lymph glands in the wild-type release cells into the haemocoel in two

Fig. 62. Horizontal section in the anterior region of a lethal benign larva showing occlusion of the alimentary canal. P - proventriculus; MS - muscularis; OG - occluded gut; FT - fat bodies; LG - lymph glands. X 150.

Fig. 63. Horizontal section in the caudal abdomen of a lethal benign larva passing through a melanised structure. OG - occluded gut; FTS - free tumour cells; FT - fat bodies; CS - clumped spheroids; ML - melanised layers. X 150.

Fig. 64. Whole mount of isolated black bodies obtained from lethal benign larvae. X 80.



periods, one at the end of the second instar when the cells give rise to imaginal mesoderm and caudal lymph hearts, and the other at the end of the third instar. A description of this latter period has been deferred until now, because the cells released then exhibit characteristics whose importance can only be appreciated in relation to the phenomena we have seen in lethal-benign. The lymph gland cells, after being released, move along in the narrowest base between the dorsal abdominal muscles and the hypodermis until they reach the haemocoel of the last abdominal segment, where they accumulate along with the similar cells which had been released at an earlier period. They exhibit two curious and important phenomena.

1) Some of the hexagons show the window-like opening which was described in similar cells in lethal-benign lymph glands.

2) Some of the spheroids become transformed into spindle cells which are almost identical with those which are a feature of the antagonistic activities which lead to the formation of black bodies in lethal-malignant and lethal-benign larvae.

Oftedal (1953), who has studied the body formation in certain circle tumour stocks, seems to have overlooked these spindle cells in the wild-type material, presumably because of the small amount of control material he examined. There is considerable variation between

individuals in their frequency. Some specimens have a large number of them, as illustrated in Fig. 25; some a few, while others appear to have none at all. In all cases, however, there are spheroids which do not transform into spindle cells but show cellular alterations similar to those seen in the spheroids of lethal-malignant larvae in which the actions antagonistic to tumour growth are occurring.

We see then that even in wild-type individuals at the end of larval life the cells of the lymph glands begin to undergo changes in the same direction as those which, carried to greater lengths in the lethal stocks, lead to the appearance of either benign or malignant tumours.

DISCUSSION

The first tumour described in Drosophila 1(I)7 (Bridges 1916) was connected with a lethal. Through the work of Stark (1918, 1919a and b, 1935, 1937), the discussion was largely centred around the question of whether these tumours (given the name black or melanised bodies in this thesis) were malignant, their homology with vertebrate tumours being taken for granted. The malignancy of the tumours was regarded by Stark as established after she found that tumours transplanted to wild-type larvae caused them to die. The experiments were repeated by Russell (1940) who was unable to confirm the tumour malignancy, although she claimed to have found that the black bodies grew and proliferated in the host larvae. Hartung (1950) found that some tumour-bearing larvae of the tumour stock bw^{tu} gave rise to adults with mis-shaped appendages which he attributed to invasion by the tumours of the imaginal discs. The melanised layers of the black bodies were considered by him to act as a barrier limiting the growth of the tumour mass. This can hardly be the case, since often the melanin does not form a continuous layer around the encapsulated tumour tissue, and yet the exposed tumour tissue disintegrates along with the covered portions. Oftedal (1953) reported that tumour tissue of the tumour stock tu(2)49K:ma 49a has sometimes been found to invade imaginal discs and

that flies with one wing and a large tumour in the thorax have been recovered from stock bottles. He explained these cases either in terms of mechanical pressure or of the possible amoeboid motility of some cells of the outer layer of the tumour, which enabled them to infiltrate the imaginal buds.

The Drosophila tumours described in the literature have the histological configuration which has been described here as characteristic of the melanised or black bodies. It would seem appropriate in the future to use these terms instead of referring to such bodies as tumours. Not only does the expression "black body" describe exactly what is meant, but its use would tend to avoid confusion between such structures and true malignant neoplasms. It has become clear that these bodies are formed after the cancerous conditions have reached their end. The changes which take place and the cells which go to form the black bodies show that they have been involved in an antagonistic reaction which, although essentially different from that exercised by the giant cells in lethal-malignant, is sufficiently similar to it to suggest some underlying physiological affinity between the two processes. On the other hand, the regular destruction of the cell components of these bodies makes it impossible to regard them as direct consequences of the processes which initiate neoplastic growth. Rather they represent the degenerative phase

of tumour tissue which at an earlier stage may, or in other cases may not, have actually been malignant. It is therefore incorrect to maintain that these bodies are melanotic tumours. There is no true homology between them and any form of atypical proliferative growth either malignant or benign. Further elucidation of this point will be made in the second part of this thesis where experimental methods have given reason for suggesting that the black bodies are the result of an antibody-antigen reaction.

Our studies of the origin of the black bodies makes it quite clear that they are the final result of a sequence of processes initiated by the lymph glands. Thus this brings no confirmation of the views of Russell (1940), who suggested that they originate from imaginal cells. Our data agree in the main with the opinion of Oftedal (1953). In considerable contrast with the black bodies the mixed cell-type carcinoma described in lethal-malignant, shows features apparently homologous with those of mammalian malignant growths. This is most clearly illustrated in the accounts of primary and secondary aggression resulting in the necrosis of the infiltrated organs. The definite manner by which the spread or metastasis of the tumour takes place is also similar to that in mammals. In Drosophila, in which the anatomy of the body is far simpler than in mammals, processes of metastasis occur

in a more clear-cut form than they do in the lymphatic or blood stream embolism of human carcinoma or sarcoma. It seems clear that the three events of primary and secondary aggression and metastasis are features common to all malignant tissues irrespective of the class of host. One may therefore be ready to admit the existence of full homology between the lymph gland tumour in Drosophila and vertebrate malignant neoplasms.

The relatively simple organisation of Drosophila may render this malignant tumour particularly favourable material for studies on the physiological and cytological steps which occur during the transformation of normal to malignant tissues. It is usually considered that a normal tissue passes through at least two such steps before it becomes fully neoplastic. In the first step there are processes operating within the tissue itself whereby its cellular components acquire cancerous tendencies. In Drosophila the symptoms of this step seem to be the appearance of more hyperchromatic nuclei and of a subsequent tendency for cellular proliferation. The second stage is a preparation for aggression and consists of processes which rupture the tumour mass so that the cells start to migrate and attain direct contact with the surrounding organs. The behaviour of the thread cells in Drosophila suggests that they play an essential role in this step, since it is as a

consequence of their elaboration that the outer epithelium of the gland is ruptured.

The discovery of this tumour, the appearance of which is directly controlled by a genetic factor which follows a clear-cut sex-linked inheritance, lends support to those theories which emphasise the importance of the genetic constitution and the causation of malignant diseases. Moreover the different antagonistic processes we have described, such as those displayed by the platelet cells, or the other unanalysed reactions which cause the aggregation and capsulation of tumour cells, give some useful indications as to why the cancer phenotype does not, in many cases, follow a classical gene segregation ratio. It is clear that we might have carriers of cancer-producing lethals which yet remained phenotypically normal, since the potential neoplasm might have been suppressed by such counteracting processes. Again, if the neoplasm grows for some time but later the antagonistic processes become more strongly developed, we should expect a spontaneous regression of the tumour, a condition which, of course, is well known to occur.

It may be pointed out that the tumour described here in which the antagonistic reactions are well marked is one which is controlled by only a simple gene or factor. It may be that such antagonistic reactions would be less marked or less effective if the

genotype contained several factors all acting towards the production of tumour tissue. Students of the genetics of tumours in mammals usually emphasise that complexes of factors are required to endow the animal with tumour susceptibility. It may be that in Drosophila multiple factors will be needed to maintain cancerous conditions as permanent as those in higher animals.

The occurrence of two counterbalancing tendencies, one towards the formation of neoplastic growths and the other opposed to it, is one of the most remarkable features of the lethal-malignant larvae. It is believed that this fact has considerable general significance. This is suggested by the occurrence of a somewhat similar situation even in normal wild-type larvae. In these, as we have seen (page 75), spindle cell formation occurs at the very end of the third larval instar. In the tumour stocks such cells are part of the antagonistic system. Their occurrence in wild-type suggests that there also the antagonistic system is developed, though very weakly. It is natural to suppose in these circumstances that there is also in the wild-type a certain weak tendency towards neoplastic growth. We arrive then at the conception of two counteracting systems both of which occur in wild-type larvae as well as in the malignant ones, though in the latter they are both developed in a very

much more strongly expressed form. One must imagine that in the wild-type both these systems are under polygenic control by genes distributed throughout the whole chromosome complement. The switch towards the development of actual neoplasm could then occur either by the mutation of a gene in the tumour-promoting system to a stronger allelomorph, or by the mutation of one in the tumour-opposing system to a weaker form. In this way we can conceive of the tumour-producing mutations as something within the natural system of the organism instead of them appearing as something foreign and extraneous to it.

The facts available so far do not allow one to decide whether the lethal-malignant mutation is one which increases the potency of the tumour-promoting tendencies or one which reduces the capacity of the tumour-opposing ones.

The peculiar phenomena shown in the testes of lethal-malignant larvae cannot yet be properly understood. It is suspected that the testes tumours arise from special cells, lying at each end of the gonad, which have been overlooked in previous investigations of its structure. Further studies of wild-type testes are being made to see if any such cells can be located. Further studies are also being carried out on the growth and metamorphosis of the gonads in order to try to clarify the processes leading to the appearance of fully matured sperm in gonads which remain larval in their

main configuration.

The causes of death in lethal-benign and similar larvae.

Russell (1940) studying the stock 1(I)7 which is characterised by the appearance of numerous black bodies, considered that these bodies themselves were not the direct cause of the lethality but that death was brought about by starvation as a consequence of the occlusion of the larval gut, which occurred at 65 hours after hatching. Beadle, Tatum and Clancy (1938) had shown that larvae deprived of food before the "70-hour change" are unable to pupate. However, the latter authors reckoned larval age from egg laying, while Russell reckoned it from egg hatching. Thus the 70-hour change of Beadle, Tatum and Clancy would correspond approximately to a 50-hour change in Russell's material and as far as this factor is concerned, the 1(I)7 larvae, which can feed up to 65 hours after hatching, ought to have been able to complete their development.

Lethal-benign shows almost the same features of tumour formation and alimentary canal abnormalities as 1(I)7. In the lethal-benign stock investigated, the occlusion of the alimentary canal occurs at about 85 hours after hatching, soon after the blood-forming organs begin the process leading to the production of black bodies. The interval between the beginning of these changes in the lymph glands and in the gut is so

short that the two effects may rather be pleiotropic manifestations of the gene than be related as cause and effect. A similar close correspondence between the times of appearance of black bodies and of the intestinal occlusion has been found in several other tumour stocks which are not described in detail in this work. It is surprising that the relation does not appear to hold in Russell's stock of 1(I)7, since in that the black bodies apparently do not become visible till the late third instar, in which case occlusion would be expected at about the 85-hour stage instead of the 65-hour stage reported by her. Even if occlusion really does occur as early as the 65-hour stage in Russel's stock, it seems doubtful whether this should in itself lead to death. Special tests have been made in which wild-type larvae have been deprived of food. It has been found that if they are removed from food at the 65-hour stage they can still pupate normally and develop into fully formed though somewhat undersized adults. It is therefore highly probable that neither in 1(1)7 nor in lethal-benign is the death of the larvae due to starvation. Another reason for seeking some other cause of death is that in both lethal stocks the larvae die at approximately 100 hours of age, while wild-type larvae which are starved before the critical period wander about for several days before perishing and thus attain a considerably longer life span.

The nature of the real cause of death in these two stocks must still be regarded as unknown.

The origin and properties of the imaginal mesoderm

It was pointed out above (page 53) that the histological evidence strongly suggests that the mesoderm of the imaginal buds is derived from the lymph glands. The evidence for this is partly that the lymph glands can be seen to liberate cells at the end of the second instar, just at the time of the appearance of the imaginal mesoderm, and partly the close histological similarity between the mesoderm and lymph gland cells.

During the first and second instar the imaginal buds are in the form of simple epithelial invaginations and show no signs of local differentiation in the form of foldings. As soon as the mesoderm reaches them these foldings begin and differentiation of the epithelium starts. One is therefore strongly tempted to suppose that the mesoderm plays a leading role in initiating the differentiation of the imaginal buds.

Direct experimental evidence of this is not easy to obtain. Vogt (1944) has obtained differentiation of the eye antennal bud taken from hosts which had not yet reached the end of the second instar, and transplanted into older larvae accompanied by a number of ring glands. It seems probable that at the time of transplantation these buds did not contain mesoderm,

though no data are given on this. There is, however, no reason why mesoderm should not have been supplied to them by the lymph glands of the host into which they were grafted and thus their behaviour provides no evidence that imaginal differentiation is possible in the absence of mesoderm. It may be remarked, however, that, as pointed out to me by Professor Waddington, in phenocopies of bithorax induced by ether treatment of the young egg, the outer structures of the metathorax may be transformed into a secondary mesothorax, but this is not accompanied by the appropriate mesothoracic mesodermal tissues in the adult. It is not at present known whether the mesoderm is always deficient in these transformed mesothoracic buds or whether the phenocopying treatment acts differentially on the epithelial and mesodermal components of the bud. An investigation of this has been begun. It is hoped also to study the whole matter of the relation between mesoderm and the rest of the imaginal bud by the use of the lethal stock "no-discs". In this, as shortly mentioned above (page 61), there is a total absence both of the imaginal discs themselves and of the lymph glands. In the normal course of events the larvae of this stock do not pupate. If, however, an imaginal bud from some other stock is injected into such a larva, accompanied by one or more ring glands from a larva capable of pupation, it may be that changes in the direction of imaginal

differentiation will be stimulated in the transplanted buds. Now since the host larva would lack any lymph gland, it would not be able to provide mesoderm for a transplanted bud which did not already possess that tissue. It is hoped that in this way one could study the capacities of the epithelial component of the bud isolated from any mesoderm.

In buds of more normal stocks which contain both mesodermal and epithelial components, the first sign of differentiation is the appearance of a characteristic pattern of folds which correspond to the later segmentation of the organ which the bud will form. In these folded buds the groups of mesoderm cells fit snugly into the folds of the epithelium. This must be the result of mutual interactions between the two tissue components. In the first place it can scarcely be supposed that the mesoderm cells, when liberated from the lymph gland, are already determined as to the organ which they will later form. It can hardly be doubted that they reach the various imaginal buds in an indifferent condition and that it is in the first place the epithelium of the bud which imparts its organ-specific character to the mesoderm which joins it. After this step has been taken, it might continue to be the epithelium which played the leading role in determining the pattern of folding, but there is no a priori reason why the mesoderm should not, at this

stage, take over the main responsibility for later events. No decision as to this can be taken from the study of wild-type material in which the proliferation of the mesoderm into a series of heaps parallels exactly the folding of the epithelium. However, both in lethal-malignant, and more particularly in lethal-no-differentiation, we find a tendency for the mesoderm to proliferate to a greater extent than corresponds with the folding of the epithelium, so that the latter either becomes raised into a flat and unfolded shape or even becomes ruptured. In these cases then it is clearly the mesoderm which is playing the dominant part and impressing on a relatively passive epithelium the results of its own abnormality.

The over-proliferation of the mesoderm could be regarded in either of two ways. We might suppose firstly that some factor is stimulating it to more rapid cell division than normal, or, secondly, we could suppose that the mesoderm has an inherent tendency for rapid proliferation which, in the wild-type, is held in check by some opposing tendency. Since the over-proliferation occurs in lethal stocks which are, on the whole, characterised by degenerative changes, it seems simpler to adopt the latter hypothesis and to suppose that a normal inhibiting agent has degenerated rather than that a normal stimulating agent has become more powerful. According to this

hypothesis, we should suppose that the mesoderm cells which reach a particular imaginal bud would acquire from the epithelium the characteristics of the corresponding organ. Initially this characteristic amounts only to a certain pattern of reaction to the growth-inhibiting factor such that certain parts of the mesoderm proliferate into heaps, while other parts are inhibited in division. As a consequence of this differential growth of the mesoderm, the epithelium becomes folded into a corresponding shape.

The bud differentiation hormone.

It is natural to imagine that the agent which we have postulated as inhibiting the proliferation of the mesoderm is actually a hormone released from some glandular structure within the animal. The whole subject of hormones in insects in general and in Drosophila in particular is still in a very confused state (see reviews by Wigglesworth, 1945, and Seidel, 1952). In Drosophila there is certainly a hormone which causes puparium formation and which, as Hadorn reported in 1937, is secreted by the ring gland, a small endocrine structure situated dorsally between the two hemispheres of the larval brain. Transplantation of several ring glands into younger larvae induced precocious puparium-formation, but no further

development occurred (Hadorn and Neel, 1938). The imaginal differentiation of pupal abdomens is completed if the anterior part is removed about 20 hours after puparium formation, but not if it is removed earlier (Bodenstein, 1938, 1939). That suggests that the imaginal differentiation requires a second factor over and above that which produced puparium-formation, and that the second factor originates somewhere in the anterior part of the animal. Bodenstein (1939) originally believed that this factor was the thoracic system of tracheae, the effective agent being, in fact, the oxygen which this system makes available to the developing tissue. More recently the opinion seems to be gaining ground that the second factor is also a hormone produced by the ring gland (cf. Seidel, 1952; Vogt, 1946; Bodenstein, 1947). Unpublished work in progress by the author, however, suggests that the migration of mesoderm cells from the thorax plays an important part in the development of the imaginal abdomen.

In most other insects the hormone which brings about larval moulting or puparium-formation is produced by cells in the brain, although in Lepidoptera, as shown by Fukuda and Williams, the brain operates only through the intermediary step of activating the pro-thoracic gland which in turn produces the immediately operative secretion. The "moulting hormone" produced

either by the brain or the pro-thoracic gland is normally opposed in its operation by a hormone produced by the corpus allatum. This produces what Wigglesworth names the "juvenile hormone", the action of which is to inhibit imaginal differentiation. Thus, if the juvenile hormone is present in sufficient concentration, the change produced by the moulting hormone is merely that of a larval moult, whereas if the juvenile hormone is absent, the moulting hormone alone causes imaginal differentiation to begin. In Drosophila also the corpus allatum produces a similar juvenile hormone (Vogt, 1946). This gland is, in Diptera, incorporated into the ring gland, which also possesses a further component, the corpus cardiacum, which seems to cooperate with the corpus allatum in producing a hormone which operates to suppress imaginal differentiation. Thus the one structure, the ring gland, appears to produce both the moulting hormone, which causes shedding of the exocuticle, and the juvenile hormone, which inhibits the moulted animal from developing into the imago.

There has been no previous study which gives any direct information on the relation between these hormones and the proliferation of the mesoderm in the imaginal buds of the third instar larva. In lethal-no-differentiation, in which this proliferation is markedly abnormal, the larval ring glands appear perfectly

similar to those of wild-type individuals. On the other hand, we find that both in this stock and in lethal-malignant abnormally high rates of imaginal mesoderm proliferation tended to follow degenerative changes in the lymph glands. It is, of course, possible that both genes have pleiotropic effects on these two different organs, but this seems a somewhat improbable assumption. A more simple explanation would be the hypothesis that the lymph glands are the source of the hormone which tends to inhibit the proliferation of the imaginal bud mesoderm. The histological appearance of the lymph glands is not at all inconsistent with the suggestion that they are endocrine organs. We may therefore suppose that the early and far-reaching degeneration of the lymph glands in lethal-no-differentiation leads to a cessation in the production of its hormone and thus to a release of the imaginal mesoderm from inhibition. In lethal-malignant and lethal-benign larvae similar changes in the mesoderm sometimes occur at later stages in larval existence and can also be attributed partly to a reduction in production of the mesoderm-inhibiting hormone and partly perhaps to the mere extension of the length of larval life owing to the failure of pupation. In these two tumour lethal stocks the imaginal mesoderm, after undergoing abnormal proliferation, suffers degeneration and cellular death. This may

probably be attributed to the occurrence of changes similar to those which antagonise the development of the neoplasm. The mesodermal and neoplastic cells are both derived originally from the lymph gland and therefore are rather similar to one another. The hormone which has just been suggested to originate from the lymph gland, may be spoken of as the "bud differentiation hormone" since the character by which it is recognised is its action on the proliferation of the mesoderm in the imaginal buds during the third instar. Its relation to hormones which operate on the development of the imaginal organs during the pupal period remains for investigation. It may be pointed out that, although Bodenstein considered that the same ring gland hormone which operates during metamorphosis also affects the early differentiation of the buds, his evidence was defective in one respect. It is known that the ring glands undergo remarkable metamorphic changes soon after puparium-formation, yet the fate of the ring glands which Bodenstein transplanted into young larval hosts was not reported. It is important to determine whether they metamorphose or remain histologically unchanged.

Hormone requirements of the different discs.

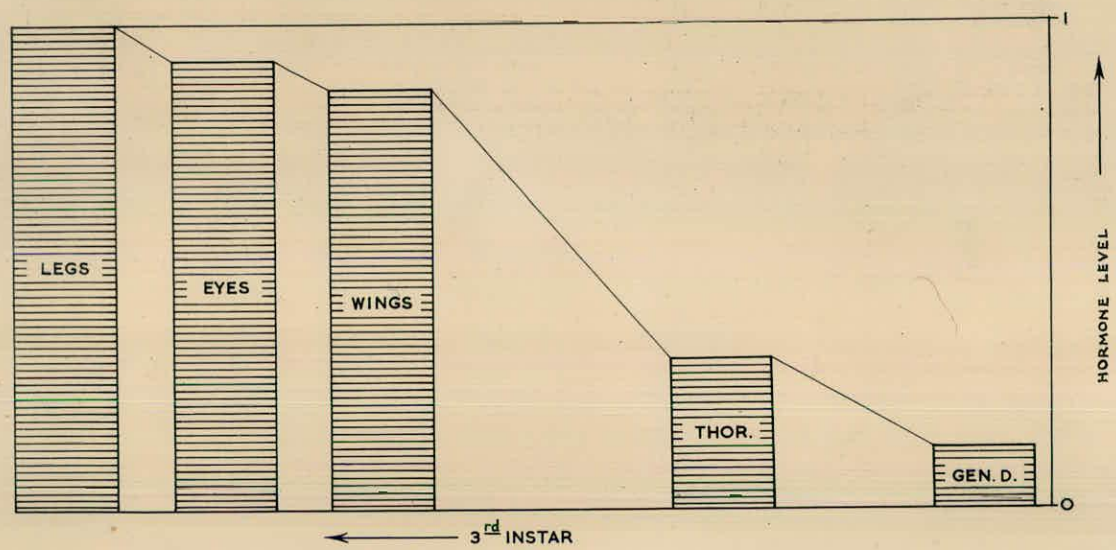
The graded patterns of damage exhibited in the pupae of lethal-no-differentiation suggest a graded series of responses by the various organs to some

common stimulus. Since the abnormalities do not arise first at the pupal stage but are derived from earlier anomalies in the imaginal buds of the larva, we may discuss the patterns of damage in terms of behaviour of the imaginal bud mesoderm. The situation could be easily understood if we suppose that, although the postulated bud differentiation hormone operates on all the discs, they differ in the threshold of hormone concentration at which the mesoderm-inhibiting action is effective.

Certain buds or parts of buds may be grouped together in their behaviour. Thus, although the dorsal and ventral parts of a thorax are formed from parts of different imaginal discs, yet they are always both present or neither of them is. So far no case has been found where either a sternum or a tergum occurs alone. Thus the proximal parts of the discs of both the ventral and dorsal sets will be treated together as corresponding similarly to the same stimulus. Again, all three pairs of legs behave similarly to each other and the whole set of them can be regarded as responding as one unit.

We can represent the requirements of the different discs by means of a diagram such as that in Fig. 68. In this the horizontal line marked 0 represents the hormone level at the early hours of the third instar when differentiation begins. During the course of the instar the hormone level in wild-type individuals

REQUIREMENTS OF THE DIFFERENT DISCS FROM THE MAXIMUM DIFFERENTIATION HORMONE THRESHOLD.



would rise to something greater than that indicated by the line I, whereas in lethal-no-differentiation larvae, at the best it only attains this level and sometimes falls below it. The hormone requirements of the discs developing into the various organs can be assessed by considering the frequency of the corresponding defects. The diagram is based on a study of some 150 pupae. In all these the legs were defective. Thus the hormone level never reached the threshold required to control the mesoderm proliferation in these imaginal buds. Eye defects were almost as common, leg defects alone being rare. Thus the eye threshold must be near the maximum attained. Again, most of the individuals showed legs, eyes and wings all affected, from which it follows that the wing threshold is only slightly below that of the eye. A smaller but appreciable fraction had no thorax in addition to the leg, eye and wing deficiencies. The thorax threshold must therefore be fairly considerably below that of the wing. The rarity of the type with an abnormal genital system in addition indicates that the threshold for the genital disc is considerably below that for the thorax.

The hormone concentration attained in a developing lethal-no-differentiation larva must be affected by the time at which the lymph glands begin to degenerate. Since the early signs of the later pupal deficiencies

can be recognised in the imaginal buds of the larva, it was possible, by a study of the numerous series of sections which had been prepared, to discover how far there was a relation between time of degeneration of the lymph gland and the nature of the imaginal buds affected. It was found that larvae in which the lymph gland showed signs of considerable damage at a stage earlier than 15 hours after the beginning of the third instar always showed abnormalities in those parts of the imaginal buds which would give rise to the thorax, whereas when the lymph gland degeneration did not occur till 24 hours or more after the beginning of the third instar, these portions were normal and only the imaginal rudiments of the wings, eyes and legs were affected. In the diagram in Fig. 65 the thorax threshold has been placed at a distance to correspond to 15 hours, while that of the wings is placed at a distance to correspond to 24 hours. It is apparent that the rise in hormone concentration between these two periods must be more rapid than either before or after, the curve of hormone production against time being apparently S-shaped.

It will be seen that in the hypothesis advanced here the mesoderm is supposed to react differentially to the hormones present and it does so in two different ways. In the first place, within any one imaginal bud the mesoderm proliferation is more strongly

inhibited in certain places than in others, the former developing into thin regions of mesoderm which correspond to the downwards folds of the epithelium, while the latter form thicker lumps corresponding to the bulging of the epithelium towards the internal cavity of the peripodial sac. Secondly, the mesoderm of each bud as a whole has its characteristic hormone requirement, as exemplified in the thresholds which we have just discussed. The relation between these two types of differential behaviour is not yet clear. As was pointed out earlier (page 88), it is to be presumed that these regionally different characters of the mesoderm have been impressed on it at an early stage by the imaginal bud epithelium with which it becomes associated. This epithelium has, of course, been derived from different regions of the embryonic hypoderm, which have been invaginated in the early formation of the buds.

It is curious that the hormone thresholds we have been led to postulate run in the opposite direction, although, as far as the data go, in the same order as those deduced by Bodenstein in another connection. In order to compare the responsiveness of the various kinds of imaginal buds towards the same level of the hormones concerned in metamorphosis in pupation, he transplanted a leg, eye and genital disc from the same donor larva together with ring glands into an adult host. He

found that under conditions in which the leg discs were able to differentiate to a complete imaginal organ, the eye disc only reached the first stages of pigmentation and the genital disc failed altogether. This would seem to indicate that the genital disc has the highest requirement for hormone while the leg disc has the least. In lethal-no-differentiation the situation is exactly opposite in that it is the legs which are most frequently abnormal and the genital system which is most resistant to damage. No adequate explanation for this difference can be given at present but the facts would certainly seem to indicate that the hormones operative in Bodenstein's cases, which were presumably given off by the transplanted ring glands, are not the same as those which control the mesoderm proliferation, which is deranged in lethal-no-differentiation. It is perhaps worth remarking that the difference in the order of ease of development as exhibited in Bodenstein's transplantations and in the pupae of lethal-no-differentiation is a matter of the observed frequencies with which different organs are found and remains a real difference which cannot be avoided whatever hypothesis one may make as to the stimulatory or inhibitory action of the hormones concerned.

SUMMARY

1. A detailed morphological and histological description is given of three recessive sex-linked types in *Drosophila melanogaster*. These are lethal-malignant, lethal-benign and lethal no-differentiation.
2. A very important part in the development of all three phenotypes is played by the lymph glands or blood-forming organs which lie as a series of paired bodies on each side of the dorsal blood vessel of the larva. The histology and development of the lymph glands in wild-type larvae is described in detail. The glands contain three main types of cells, namely hexagons (some of which are binucleate), platelets and spheroids.
3. In the late second instar of the wild type the lymph glands release some of their cells and these form the imaginal disc mesoderm, the lymph hearts, and other structures. The formation and the functioning of the lymph hearts formed in the posterior end of the larval body are described.
4. Wild-type lymph glands release their cells for a second time at the end of the third instar. These cells after their liberation show changes similar to, but not so marked, as those to be described in the tumour-bearing lethals. Thus the hexagons show nuclear abnormalities and some of the spheroids become transformed into spindle-shaped cells. It is argued that these cells cannot be truly regarded as blood cells, and that, in fact, no such cells exist in the *Drosophila* larva.
5. Lethal-malignant causes the development of the first true malignant tumour to be described in *Drosophila*. The tumour

appears in the late third instar and prolongs the larval life for several days. The cells of the tumour originate from the lymph glands. The processes by which the cells of these glands are liberated into the body cavity and their transformation into new cell types (thread cells, giant cells, binucleated giant cells and tripartite cells) are described.

6. The tumour cells first infiltrate the imaginal disc. They then metastasise along the ventral nerve cord, and, after arrival at the posterior end of the body, attack the fat bodies and other organs. It is, however, only in a comparatively few cases that the whole of this process is completed, the growth of the tumour being usually arrested before the fat bodies are attacked.

7. The growth and spread of the tumours is in all cases opposed by certain antagonistic tendencies. Some of these take a morphological form (various forms of encapsulation by giant and other cells). More important antagonistic activities are manifested as changes in the nature of the cells composing the tumour. Thus the hexagons undergo degeneration of which an early sign is the deposition of melanin. The spheroids become transformed into spindle cells, and groups of them become clumped together. The degenerative processes terminate in the general deposition of melanin and formation of melanotic black bodies. These black bodies are similar to the structures which have usually been referred to as tumours in *Drosophila*.

8. Tumour tissue, before it shows any sign of degeneration develops spots of black pigment when subjected to high

temperature (80-90). This reaction provides an easy method of following the spread of the metastasising tumour along the ventral nerve cord.

9. Tumour cells, whose origin cannot be exactly determined, may appear in the testes of lethal malignant larvae. They invade the surrounding germ tissue and lead eventually to necrosis of the invaded areas. Fully mature sperms may also be found in the testes of these larvae, although they do not occur till a much later stage in wild-type individuals. They eventually develop a strong basophilia and later degenerate.

10. The larvae of lethal benign develop melanotic bodies which usually float freely in the haemocoel. Their structure is similar to that described for the well-known tumour-bearing lethal 1(1)7. It is shown that the cells comprising these tumours are derived from the lymph glands and that the so-called tumour is a product of cellular degeneration and cannot justly be compared with a proliferating malignant growth.

11. The larvae of lethal benign, like those of 1(1)7, develop an occlusion of the mid-gut. The histological origin of this condition is described. The death of the larvae should probably not be attributed to starvation following the occlusion of the gut, and its cause remains obscure.

12. The most obvious effect in lethal no-differentiation is a failure of some or all of the imaginal buds to continue differentiation after the beginning of the prepupal period. This is found to be a consequence of an abnormal prolifer-

ation of the imaginal disc mesoderm.

13. It is found that there is a well-defined sequence in the sensitivity of the different organs to disturbances by the lethal no-differentiation effect, the whole group of legs being most commonly affected, and the wings the least commonly.

14. The abnormal proliferation of the imaginal disc mesoderm in lethal no-differentiation is preceded by degenerative changes in some of the cellular constituents of the lymph glands. Changes in the imaginal discs, somewhat similar to those seen in lethal no-differentiation but less marked in degree, can be found in certain individuals of lethal malignant.

15. In the discussion a number of hypotheses are advanced, which emerge from the whole body of facts derived from the comparative study of the three mutants.

(i) It is suggested that even in the normal wild-type individual there are certain tendencies towards the development of neoplastic growth which are opposed by antagonistic tendencies. Symptoms of the latter can be seen in the changes occurring in the lymph glands at the very end of larval life in the wild-type. It is argued that the development of an actual neoplasm can be the result either of the stimulation of the neoplasm-promoting system, or of an inhibition of the neoplasm-opposing system.

(ii) The mechanism of differentiation of the imaginal discs is discussed and it is argued that the organisation of the imaginal organs is determined primarily by the

the mesoderm.

(iii) It is suggested that the proliferation of the imaginal bud mesoderm is normally held in check by a hormonal influence originating from the lymph glands. The relation between the postulated "bud-differentiation hormone" and the hormone or hormones concerned in the transition from the larval to the pupal condition cannot yet be determined.

PART II

TRANSPLANTATION EXPERIMENTS WITH TUMOUR AND ALLIED
TISSUE

PART II

TABLE OF CONTENTS

Introduction	1
Principals immunological phenomena and nomenclature	2
Immunological phenomena in the transplantation of mouse tumours	3
Insect immunology	5
Stocks and technique	8
Experimental results	10
Immunisation of malignant larvae	16
Complement-fixation reaction	19
Summary of the results	26
Discussion	27
Immunological phenomena and the development of tumours in <u>Drosophila</u>	27
The bearing of <u>Drosophila</u> tumours on the general problems of cancer	40
Summary	47
Literature cited	51

INTRODUCTION

A considerable number of transplantation experiments have been made with the lymph glands and the tissues derived from them. It was originally hoped to investigate in this way the hormonal relations which have been suggested in the first part of the thesis. It was found, however, that these tissues rarely survive when transplanted into another larva. Instead they suffer degenerative changes which will be described in this part. It is believed that these changes can be regarded as the expression of immunological reactions going on between the implanted tissues and the body fluids of the host. In the discussion of the work, therefore, the line of thought which has led from one experiment to the next will be expressed in terms of immunological concepts. The justification for this must be sought in the degree to which such concepts enable one to bring the complex body of facts into a single unified scheme.

In the first part of the thesis we found reason to suggest that we are dealing with a system of two interacting tendencies, one towards the formation of a neoplasm and the other opposing this. Both tendencies occur even in the wild-type larva where, however, the second is on the whole more powerful, whereas in the tumour stocks the balance is swung the other way. We

shall find that the transplantation experiments also suggest that, in the very late stages of larval wild-type, lymph glands exhibit antigenic properties similar to those of the neoplasms from the malignant stock.

Principal immunological phenomena and nomenclature

Substances inciting the formation of, and reacting with, antibodies are called antigens. Sera that contain antibodies as the result of the injection of antigens are called immune sera or antisera. Natural antibodies are substances found in the serum of untreated animals which are similar in their effects to the antibodies developed by immunisation. A rather detailed nomenclature has been built up around the diverse manifestations of antigen-antibody reactions, the antigens and antibodies being described with reference to the kind of reaction observed. The clumping of cells is known as agglutination and the antigens and antibodies involved are called agglutinogens and agglutinins (e.g. hemagglutinins, bacterial agglutinins) respectively.

Similarly, the antibodies causing disruption of cells (lysis) are designated as lysins (e.g. bacteriolysins, hemolysins). Precipitins are those which cause precipitation when mixed with the inciting soluble antigens. The expression passive immunisation signifies the temporary protection conferred upon an

animal by the direct administration of immune sera, in contrast with active immunisation in which antibodies are provoked by the administration of the antigen itself.

In mammals, proteins from tissues unfamiliar to the circulation (i.e. eye proteins) might behave as isoantigens and stimulate the production of antibodies if injected into the circulation of the same donor. On the other hand, foreign proteins injected, for example, into the anterior chamber of the eye do not provoke any immunity reaction. This is likely the cause of clinical success of corneal homografts in ophthalmic surgery. A skin homograft survives transplantation to the eye of a specifically and strongly immunised rabbit if it remains unvascularised (Medawar, 1948). In Drosophila, isoantigens similar to those of mammals do not normally occur. This is naturally due to the open type circulation, where all tissues are completely bathed by the circulating haemolymph. Therefore materials which stimulate an immune reaction must be considered entirely foreign to the organism and not just foreign to the haemolymph.

Immunological phenomena in the transplantation of mouse tumours

In mice, genetic studies on the transplantation of tumours have been carried out by several workers (reviewed by Bittner, 1936). Loeb (1900) inoculated

a tumour which arose spontaneously in an individual of the Japanese waltzing strain of mice. He observed that the members of this strain were all susceptible, whereas mice of the white variety were all resistant. Tyzzer (1909), inoculating a carcinoma of the mammary gland of the Japanese waltzing mice, verified Loeb's findings and added the discovery that the F1 hybrids produced by crossing the parent susceptible strain (inbred) to common mice were likewise all susceptible. Little and Tyzzer (1911) continued work on this tumour, and found that, amongst the F2 hybrid offspring of the previous cross, only a few individuals were not resistant to the transplanted tumour. They interpreted this data by applying the multiple-factor hypothesis. From the segregation ratios, in the F2, susceptibility to the tumour inoculated was found to require the simultaneous presence of a large number of dominant factors. Similar work was carried out by Strong (1922), Strong and Little (1920), Little and Strong (1924), Cloudman (1932), and others on the inbred dilute brown dba and albino strain (strain A) of mice, both of which are highly inbred and show a very high incidence of mammary gland tumour. From their work, the same results regarding the behaviour of the host towards the tumour grafts were secured. This indicates that, while a mouse is unable to resist neoplasms related to its own, it sets up an immune reaction against a foreign

tumour graft. This susceptibility difference was formulated in the genetic theory of tumour transplantation (Little and Strong, 1924), which states that the fate of tumour transplants is determined by a reaction between the host, depending largely on its genotype, and the transplanted tumour tissue controlled to some extent by its genetic constitution. The number of factors required for the propagation of a transplanted tumour is obtained from the proportion of susceptible mice in the F2 and backcross generation to the resistant stock. For example, if a one factor tumour is inoculated, the F2 generation gives 3+ : I- individuals; the backcross to the resistant stock I+ : I-. A variable number of susceptibility factors ranging from one dominant mutation (Bittner, 1932) to indefinite factorial complexes have been reported to be required by the different tumours.

Similar results have also been obtained from work on mouse leukemias (reviewed by MacDowell, 1938).

Insect immunology

In insects, transplants between genetically different individuals of the same species usually survive transplantations (Caspari, 1933). Interspecific grafts have also been carried out with a considerable degree of success. Howland, Glancy and Sonnenblich (1937), Stubbe and Vogt (1940) and others succeeded in transplanting eye discs between a number of different

species of Drosophila. Plagge (1936) transplanted testes of caterpillars between members of different genera and different families, as from *Plodia interpunctella*, *Calleria melonella*, *Carpocapsa pomonella*, *Ptychopoda seriata* and *chrysitis* larvae and pupae into *Ephestia* pupae. The transplanted testes not only survived but developed normally in the foreign host.

In contrast with the situation in vertebrates, where immune reactions are stimulated against transplants of the same species arising from antigenic differences between host and graft, the results mentioned above show that insects behave rather passively towards foreign grafts. This might be attributed either to a lack of antigenicity of insect tissues in general, or to a failure of insects to produce antibodies to materials which are antigenic in vertebrates. The former possibility is unlikely, since extracts of insects have been shown to be strongly antigenic for rabbits (Cumley, 1939; Caspari, 1950; Fox, 1949a; Fox and Thomas, 1953a). The second possibility was investigated by Bernheimer, Caspari and Kaiser (1952). They studied directly the ability of insects to produce antibodies to materials known to be antigenic in higher animals. Results indicated that, after the injection of various antigens (*E. coli*, strain B; Coliphage T2; Streptolysin O; and human erythrocytes), the haemolymphs obtained from the injected caterpillars and pupae did not differ significantly from haemolymphs of

the control. From this it was concluded that insects at any stage of their development are irresponsive towards antigenic materials.

The same conclusion might be drawn from the apparent lack of any immunological incompatibility between the host and the grafted tissues in the numerous experiments which have been made in Drosophila following the original transplantations of eye discs carried out by Beadle and Ephrussi. However, it must be pointed out that the vast majority of such experiments have dealt with transplantations made into late third instar larvae so that little time was available for immunological reactions to develop and manifest themselves. We shall find that transplantations of the lymph glands or derived tissues produce strong reactions and, in spite of the failure of previous attempts to detect antibody formation in Drosophila, it is thought that these reactions can be attributed to immunological phenomena.

STOCKS AND TECHNIQUE

Two of the three lethal stocks described before were used - lethal-malignant and lethal-no-differentiation. According to the aim of the experiment, they have been used both as donors and recipients. The control stock is an OrK strain which has been inbred for a large number of generations and was obtained from the Institute of Animal Genetics, Edinburgh.

Transplantation and injection were the only operative methods used. The equipment designed by Beadle and Ephrussi (1935) was used with some modifications. In particular, a micrometer head was attached to the micro-syringe. This proved essential especially when young larval stages had to be treated, as the amount of injected fluid or emulsion could be precisely regulated.

Injections were carried out under semi-sterile conditions. Donor larvae anaesthetised in a glass tube etheriser were passed through saline solution, 70% alcohol and distilled water before being dissected, in order to wash out completely any trace of ether, which may act as a fixative. Tissues to be transplanted were dissected out in Drosophila Ringer, then transferred to a clean watch glass, containing the same solution. The watch glass was kept well covered in a dry cool place. Transplants remain alive for several hours.

The recipient larvae were anaesthetised under very carefully controlled conditions. Treated hyper-etherised larvae failed regularly to recover, and often

presented unfavourable material for transplantation purposes. This is due to the fact that they contract to such an extent that the internal viscera protrude as soon as the injection needle is introduced. However, successful results were obtained from adequately etherised material. This is secured by administering the anaesthesia very gradually. Thus a very small amount of ether was used in the etheriser. Larvae were placed on a clean slide which fits by one end to the cork stopper. Soon after the larvae stop moving they are passed quickly through 70% alcohol, then stretched over a sterilised slide coated with a fine film of saline solution. The latter evaporates, leaving a fine layer of salt which fixes the material firmly on the operating field. Injections done through the dorsal body wall of the larvae resulted in fatal injuries to the dorsal blood vessel. In addition, this site of inoculation was resisted by the dorsally flowing body fluid. The most satisfactory place for injection was found to be the ventral side of the last abdominal segment. Keeping the cephalic end of the material towards the operator, the stream of haemolymph which flows ventrally and posteriorly facilitates the absorption of the injected organ. A very low rate of mortality, not exceeding 5%, was generally obtained. It is advantageous to starve the larvae for several hours before they are operated on; starved larvae

recover more quickly from the anaesthesia, and the risk of infection is reduced, since the slightest injury to the alimentary canal of a fully fed animal results in contamination of the haemolymph with yeast cells.

Injected larvae were not helped to detach from the slide until the wounds completely healed up. This is shown by the appearance of a black scar in the place of the injection. They are finally transferred to fresh food vials and kept at a constant temperature of 25°C.

In injection with the neoplasm, swelling tumours with branching outline were used after having been isolated from larvae aged one day after the end of the third instar. The standard dose consisted of one pair of tumours. The tumours, after transfer to *Drosophila* saline, sometimes break down to a very thick emulsion of tumour tissue.

For histological investigations, hot Bouin was used as a fixative and Peterfi's celloidin method of double embedding was applied. The 5 μ sections were stained with Delafield's haematoxylin with no counter stain.

EXPERIMENTAL RESULTS

Experiment I

Lymph gland carcinoma from lethal-malignant larvae were injected into mid-third instar larvae of OrK and lethal-no-differentiation. Later the injected dose appeared as black bodies floating freely in the

haemocoel. Morphologically and histologically these are of the same type as the pigmented bodies usually called melanotic tumours in Drosophila.

In order to follow the sequence of events in which the injected cells were involved, larvae given the same treatment were fixed at successive periods before the black bodies became visible. The injected doses comprise both spheroids and hexagons. In both kinds of cell, the changes in cell morphology and nuclear appearance which usually lead to malignancy are known. Any structural alterations which could be identified histologically would therefore be attributed to the stimulated antagonistic reaction. Agglutination of the spheroids was striking; small groups of cell-agglutinates appeared scattered in the haemocoel, and later condensed to form larger masses. At the same time a considerable number of cells transformed to spindle cells, which clustered to encapsulate the agglutinated masses. Melanisation and lysis followed, preceded by nuclear breakdown. After the black bodies reached their final stage of formation, sections showed both phenomena occurring in an exaggerated manner, so that the agglutinated masses lyse completely and the spindle cells become deeply melanised. These changes in the injected material are identical with those reported earlier in connection with the formation of the black bodies in the two tumour stocks. Both phenomena were,

however, far more pronounced than in any of the same structures described in lethal-benign or lethal-malignant larvae. This difference has been used in subsequent experiments to distinguish between the injected neoplasm and the larva's own neoplasm when both are antagonised by the same host.

Experiment 2

a) Blood-forming organs from control late third instar larvae were injected into control early third instar larvae. Results: A day after the experiment an immune reaction was surprisingly manifested and the injected normal organs were thrown down melanised. Histologically these are a duplicate of the antibody-antigen compounds produced by injected neoplasm.

b) Blood-forming organs from control early third instar were injected into sister larvae of the same age and from the same culture (this is secured by having a larval collection period of not more than 15 minutes) . Results: The recipients were at first normal, but, as at the end of the third instar and within a short period, the injected doses were thrown down melanised.

The above results seem to indicate that, by the end of the third instar, antigenic tendencies of major importance become characteristic of normal lymph glands. Confirmation of this has been sought in various ways. It seems reasonable to suppose that the antigenicity which develops is connected with a tendency for the lymph

gland tissue to exhibit malignant tendencies. Attention has been drawn in the first part of the thesis (page 41) to a simple test of malignancy which depends on the behaviour of the tumour cells towards a high temperature. If by the end of the third instar wild-type blood-forming organs become antigenic because of the development of malignant tendencies, then they should react positively to the high temperature reaction and hence show a black spotting when suitably treated. Furthermore, by the use of such a treatment one should be able to determine accurately at what time in the third instar this physiological change in the lymph glands occurs, and the reaction may be used to follow the later changes in the behaviour of the tissues.

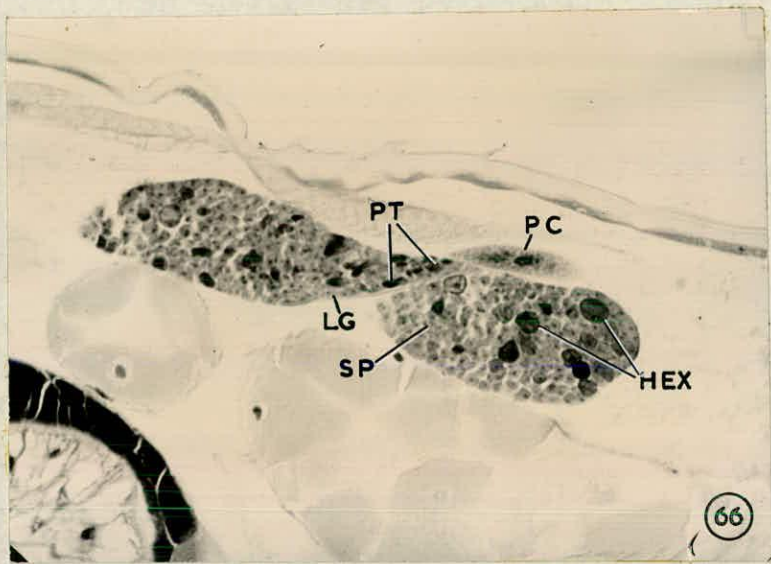
Wild-type larvae younger than 80 hours reacted negatively to the high temperature reaction. Two hours later (i.e. as soon as the larvae leave the food) wild-type larvae treated with a hot fixative show their lymph glands sparsely spotted. The dark dots were confined solely to the glands and were not found in other parts of the body. In 85-hour larvae the reaction was more vigorous and the lymph glands, particularly the first pair, had increased considerably in size. In 87-hour larvae the lymph glands are irregular in outline instead of oval. By this time the glands have ruptured and streams of cells (presumably of potentially malignant character) are flowing both

posteriorly and anteriorly along the dorsal and dorso-lateral sides of the glands. Following high temperature treatment the spotting is no longer localised strictly in the glands, but tiny black dots can be seen over a considerable area on both sides of the glands which are themselves heavily pigmented. No spotting was present in the area around the ventral nerve cord. In still later stages, from 90 hours until the formation of puparia the reaction had disappeared again and no spotting of any kind was found.

The timing just given may not coincide exactly with that obtained in other strains of wild-type *Drosophila*, since developmental rates may vary considerably, although the various features of the sequence of events remain unchanged.

Specimens from every stage in this sequence were studied microscopically. It was interesting to find that in 82-hour old larvae the hexagons in the lymph glands are melanised. The formation of melanin in these cells is not a normal functional activity of a healthy cell; it is an indication of cell degeneration or even a post-mortem event. As we will show later (Page 19), it is probably produced by an immunological reaction involving something akin to complement. The remaining two types of cells in the glands (platelets and spheroids) appear quite normal at this time except that they are proliferating rapidly. Their multiplication is responsible for the large size attained by the glands in slightly later stages. Fig. 66 shows a well-

Fig. 66. A section in the anterior pair of 82-hour old
wild-type lymph glands (LG). HEX-melanised hexagons;
PT- platelet cells; PC - spheroids. x 100



well-differentiated preparation from this stage. The hexagons appear dark owing to their content of brown melanin. The platelets are highly basophilic and are present in larger numbers than in earlier stages. The spheroids are distinguished by their faintly-stained cytoplasm and the deeply stained nuclei. Mitotic figures with spindles are frequent amongst them. The epithelial wall of the gland is intact at this stage. Sections through later stages show that the proliferation of the platelets and spheroids causes the rupture of the outer epithelium of the gland with the result that the dividing cells are released into the body cavity in the neighbourhood.

Sections through very late larvae in the stages which are negative to the high temperature reaction showed that the melanised hexagons no longer existed in the glands . They appeared to be thrown out into the body cavity , where they disintegrate. The glands at this stage, therefore, show little signs of the events which have recently taken place. The spheroids which have been released into the body cavity and also those remaining in the glands , showed some nuclear distortion. Some of them have already transformed into spindle cells and intergrades between normal spheroids and spindle cells were of frequent occurrence. It is these final stages, after the transitory appearance of the melanised hexagons, which represented the control conditions in the first section of the thesis and where the cause of spindle-cell formation was rather obscure.

The melanisation of the hexagons and the transformation

of the spheroids into spindle cells are changes of the same kind as those seen in the injected neoplasms of Experiment 1, of course, much milder in degree. We have therefore three reasons for supposing that within the lymph glands of late wild type larvae changes take place which tend in the direction of the development of malignancy of the kind which is so much more strongly developed in lethal malignant. These reasons are:- Firstly, the formation of black bodies following the injection of normal control lymph glands; secondly, the transitory positive reaction of wild type lymph glands to the high temperature treatment; and thirdly, the melanisation of the hexagons and spindle transformation of the spheroids which, as shown from Experiment 1, is due to stimulated immune reaction.

Immunisation of Malignant Larvae.

Experiment 3.

50 lethal larvae were injected with the neoplasm at the age of the 15 hours in the third instar. An immune reaction against the injected dose was visible roughly at the end of the same instar, the injected cells being thrown down as melanised black bodies. Some larvae were fixed at this stage while the remainder formed puparia a little earlier than $1\frac{1}{2}$ days after the normal puparium-formation time in control stocks.

Microscopic investigation showed that the injected dose behaved similarly to that in Experiment 1. It also revealed the remarkable fact that there was an antagonistic reaction against the host's own blood-forming organs.

There was no cellular proliferation within the glands such as that seen in untreated lethal malignant larvae. There was in consequence no massive release of tumour cells and no aggression against other tissues such as the imaginal buds or fat bodies. The nuclei of the cells were highly distorted. The larvae did not show the black spotting following high temperature treatment such as is characteristic of the lethal malignant type.

These facts suggest that the injection of the neoplasm early in the third instar has brought about a state of active immunisation which has not merely caused the production of antibodies which react with the injected cells, but has brought the host into a state in which it responds much more quickly and effectively towards subsequent introductions of the same antigen. Thus when the host larva's own neoplasm begins to develop later in the instar the body will immediately react against it and the development of the neoplasm will be suppressed.

Experiment 4.

Similar injections of neoplasm were made into rather older lethal malignant larvae ($4\frac{1}{2}$ -day). At this stage the host's lymph glands are already beginning to be malignant and are undergoing cell multiplication. The antibody-antigen reaction (black body formation) appeared a day later. The material was fixed at this time and compared with that of the previous experiment.

The larvae showed some signs of antagonising their own tumour tissue, but the antagonistic reaction was not

so strong as that in the previous experiment when the injection was made at an earlier stage. The hexagons became rather weakly melanised and the nuclei of the spheroids tended to degenerate. Tumour cells were liberated from the glands into the haemocoel. Some of these aggregated into melanised injected cells, while the majority agglutinated separately into large groups. A good many of the liberated cells acquired a spindle shape and were gathered sometimes into thick bundles.

Experiment 5.

Similar injections of neoplasm were also made into late malignant larvae. Both the donor and host larvae were aged $1\frac{1}{2}$ days after the normal end of the third instar. The experiment was carried out on as many larvae as possible. This was because many of the larvae might be expected to have already antagonised their own neoplasm before the time of injection. Furthermore, it would be particularly interesting to discover the effect of the injection in larvae in which secondary growth occurs and as was stated in the first part of the thesis, such secondary aggression occurs in only comparatively rare cases. In order to obtain as many hosts as possible with well developed tumours only larvae whose lymph glands could be seen through the transparent body wall to be considerably over-sized were used as hosts for the injection. Larvae that formed puparia soon after the operation were discarded. Those which remained in the larval condition survived for two days after the operation and were fixed and investigated.

The haemocoelae contained black bodies which can certainly be regarded as the agglutinated and precipitated injected cells. In cases where the host possessed a metastasising tumour there were indications that the aggression of the tumour had been arrested at or shortly after the time of the injection. Thus along the ventral nerve cord the hexagons showed a faint degree of melanisation. The spheroids exhibited degenerate nuclei and those free in the haemocoelae suffered agglutination. Large groups of spindle cells were abundant. Fig. 66 illustrates an antagonistic reaction where the spheroids are involved. In the material examined the fat bodies were never attacked and this is probably a consequence of the interruption of the metastasis of the tumour.

Complement-fixation reaction.

In the two previous experiments the injected doses of the neoplasm were, without exception, thrown down as antibody-antigen agglutinates with an additional reaction leading to lysis and melanisation. Yet, although the neoplasm of the larvae showed nuclear distortion, agglutination and spindle-shape formation, both lysis and melanin deposition were to a remarkable extent suppressed. This may suggest that the two latter phenomena are carried out independently by a complement naturally occurring in the haemolymph.

Complement.

For bactericidal and haemolytic reactions of immune sera, as well as for other cytotoxic effects, there is needed in addition to antibody, a naturally occurring

substance or group of substances from the serum variously collected as complement. For example, if the complement of a serum haemolytic for erythrocytes is removed or destroyed it will be found that the serum no longer produces lysis when added to the sensitised cells. Antibody is still present and active, however, as is shown by the fact that such a serum will still agglutinate the cells. If fresh normal serum, often even of a different species, is added to the mixture of the complement-free antiserum and cells, lysis may be obtained to a degree indicating that the destruction of the complement did not decrease the amount of the antibody present.

The characteristic of complement to be bound by the aggregates (precipitates, sensitised cells) formed through the interaction of antigens and antibodies is the basis for a frequently used serological test. In this complement-fixation test, introduced by Bordet and Gengou (1901), antigen and antibody to be tested are mixed with fresh normal serum and after incubation haemolytic immune serum and corresponding red cells are added as indicator for the presence of complement. If an immunological reaction takes place in the first stage, complement is fixed or removed from the mixture, and haemolysis of the agglutinated erythrocytes is prevented, completely or in part according to the intensity of the reaction. In general the reactions run parallel to precipitin reactions but complement fixation, in certain cases, gives positive results in the absence of visible precipitation. Complement-

fixation tests have a widespread application in vertebrate serology, for example, the Wassermann reaction for the detection of syphilitic reagin in the serum of syphilitic patients is simply an application of the complement-fixation technique.

We have suggested the hypothesis that in Drosophila the injected neoplasm stimulates an immune reaction. The antibody-antigen compound which appears first as an agglutinate is later found to be partially lysed and partially melanised. Although it may seem unexpected to suggest that a complement reaction occurs in Drosophila the two experiments last described involved a complement-fixation test which was carried out in vivo, and was used not to indicate that an antibody-antigen reaction has taken place, but as an indicator for the presence and effect of complement. The injection of neoplasm after the development of the host's own neoplasm can be regarded as similar to the performance of two successive injections of the same antigen. As has been described, the first inoculated dose (i.e. the injected neoplasm) shows lysis and melanisation in addition to the nuclear distortion, agglutination and spindle cell formation which occur alone in the second dose (the host's own neoplasm). One might therefore suggest that lysis and melanisation are dependent on the presence of complement in the Drosophila haemolymph

and that this is removed by being used against the first dose of the injected cells, leaving the haemolymph with a reduced quantity of complement which is insufficient to produce the same effects on the host's own antagonised neoplasm.

The following experiment was carried out to test this hypothesis.

Experiment 6.

Ten-hour old third instar lethal no-differentiation larvae were immunised against the neoplasm. After the immune reaction appeared they were re-operated and given a second dose. A day and a half later they were fixed with only the first dose melanised. Microscopic examination supports the previous conclusion.

Two different processes, therefore, operate to produce the melanised bodies. The antibody is concerned with agglutination and spindle cell formation, complement, on the other hand, produces lysis and melanisation of the sensitised cells. While lysis involves the agglutinated spheroids, melanin deposition only takes place in the spindle cells. It is, however, not unexpected that complement could affect these two different substrates in different ways.

Experiment 7.

Lymph glands from 75-hour old wild-type larvae were injected into ten-hour old third instar malignant larvae. The recipients now behaved just as wild-type larvae would have done, and the injected glands were thrown down by the

end of the third instar. The lymph glands of the host larvae never developed into a malignant state, there was no release of cells into the haemocoel, but instead an early antagonistic reaction (melanisation and lysis) occurred within the glands themselves. Puparium-formation was not delayed more than a day and a half beyond that of wild type.

Experiment 8.

Lymph glands from 85 hour old wild type larvae were injected into early third instar larvae of the malignant stock. These doses of injection comprise only malignant spheroids and were obtained from larvae whose sisters were positive to the high temperature reaction. Lymph glands of that stage, as verified histologically, showed hexagons dead and the spheroids multiplying. Results obtained showed no significant difference from those of the previous experiment.

Experiment 9.

Following the immune reaction in which the blood-forming organ components in control larvae were involved, anti-neoplasm antibodies are expected to occur in normal haemolymphs.

Haemolymphs extracted from very late third instar OrK larvae due to form puparia were injected into mid third instar malignant larvae. The existence of antibodies

in the injected fluid would be revealed by its direct passive immunisation or passive sensitisation, both of which would lead to regression of the host's tumour mother organ. The larvae were fixed one and a half days after the treatment.

Results: The behaviour of the host's lymph glands was identical with that of experiment 7. This is a conclusive evidence for the occurrence of immune bodies against the neoplasm in normal haemolymphs.

The abnormal occurrence of stages beyond the primary spermatocytes in the testes of lethal-malignant larvae suggested the following experiment, the aim of which was to investigate the behaviour of larvae towards injected doses of sperm.

Experiment 10.

Attempts were made to inject sperm dissected out from adult flies into mid-third instar lethal-malignant larvae. In order to give heavy doses the sperm suspension was thick and appeared very sticky. It was difficult to suck it into the injection needle and when attempting to inject it into the haemocoel the sperm frequently stuck to the tip of the needle and was not released into the body of the host larvae. These difficulties caused a high rate of mortality. It was found necessary sometimes to inject whole pieces of testes, but these again had to be administered with wide needles and there was a low rate of survival after the

operations. Out of 40 larvae injected only 12 survived.

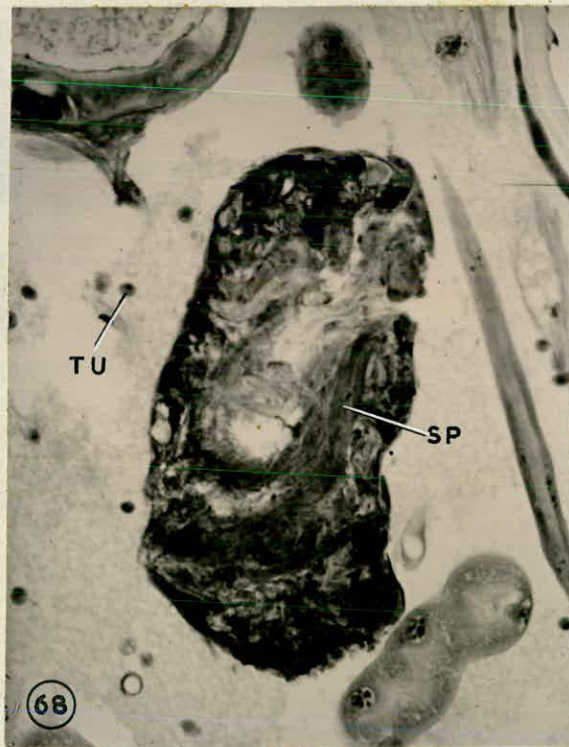
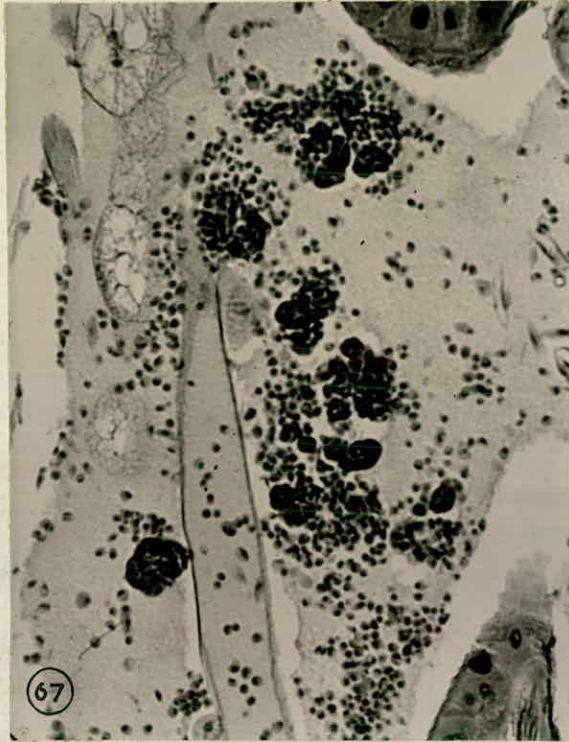
1½ days after the operation the injected sperm were thrown down melanised. Fig. 68 illustrates one of the injected pieces of testes in which bundles of melanised sperm can be seen. There is evidence of lysis and melanisation throughout all the different components of the testes.

There was also evidence that larvae immunised against sperm tended to resist lymph gland tumour tissue in a way similar to that of Experiment 7. The rest of the free tumour cells aggregated around the melanised testes masses and this may be taken as the indication of the precipitation and agglutination of these cells by antibodies. However, the host tumour cells did not show the melanisation and lysis which we have attributed to complement, this having been presumably used up in the reaction of the testes.

EXTRA STRONG

Fig. 67. Agglutination reaction in which injected neoplasm was involved as antigen. X 250.

Fig. 68. Antibody-antigen compound resulting from injecting a whole piece of mature testis into lethal malignant larvae. SP - melanised bundles of sperms; TU - lymph gland tumour cells. X 370.



Summary of the results:

- 1) Doses of the neoplasm injected into non-tumorous larvae stimulated an immune reaction and were thrown down melanised. The melanised doses are identical with Drosophila benign tumours.
- 2) A complement-fixation reaction revealed that while agglutination and spindle cell formation are attributed to antibody, both lysis and melanin deposition are complement effect.
- 3) Wild-type blood-forming organs become antigenic (malignant) at the end of the third instar.
- 4) Malignant larvae were stimulated to resist their own neoplasm by an injected dose of the same neoplasm.
- 5) Early immunisation of malignant larvae was effective.
- 6) Anti-neoplasm antibodies occur in normal haemolymphs.
- 7) Sperm suspension injected into larvae stimulated immune reaction,,the injected sperm were thrown down melanised.

DISCUSSION

1. Immunological phenomena and the development of tumours in Drosophila.

It has been clearly shown above that during the normal development of wild-type Drosophila larva, the lymph gland material, during a certain period, exhibits tendencies which, if carried further, would lead to malignancy. At the same time it calls forth antagonistic processes which have been interpreted by the hypothesis that the tissue is acting as an antigen and causing antibodies to be formed against it. The causation and significance of this change from normality towards malignancy cannot be deduced from the conditions in lethal individuals, but only from those in the wild-type, since the phenomena are not pathological in any way but are fully normal.

The mutant strains in which tumours develop, on the other hand, provide the material for studying the events which give the opportunity for these natural malignant tendencies to develop for such a long time that a definite neoplasm can be produced.

Neoplasms are, of course, harmful to the organism, yet the malignant transformation of the lymph glands in wild-type larvae must be a normal part of the processes of growth and differentiation. The larval phase in the development of Drosophila changes into that of the prepupa after the various tissues have either reached the final stage in their development or have attained the level of differentiation which is completed in the post-larval

period. The intercalation into normal development of a phase of antigenic activity and malignant tendencies may be easier to understand in the light of the following hypothesis. If a glandular tissue is stimulating or regulating embryonic processes by means of a hormone which it secretes, and if at a certain stage of the individual's life, these developmental processes have been brought to a stage of completion, then the hormone-secreting organ will be no longer required. There may, indeed, be a necessity for the hormone secretion to be brought to an end either by the abolition of the secretory organ or by its conversion into a modified structure which may fulfil some other function. A method by which this could be brought about would be that, under genic action, the secretory tissue becomes antigenic towards the individual of which it forms a part. The newly developed antigen would thus provoke the formation of antibody and the antibody-antigen reaction would lead to the abolition or modification of the organ.

We have seen in the first part of this thesis that there is reason to believe that the lymph glands produce a differentiation hormone the effect of which is primarily on the mesoderm of the imaginal discs. It appears that the hormone controls the proliferation of the imaginal disc mesoderm into heaps of different sizes, which in turn control the folding of the overlying ectoderm.

We may therefore suppose that as soon as the mesoderm has proliferated sufficiently to produce the segmentation pattern characteristic of the normal imaginal discs, the differentiation hormone will no longer be required and the site of its production must be eliminated. Investigation shows that the imaginal discs become fully differentiated at 80 hours after hatching. No further differentiation takes place in them in the remaining part of the larval life until the beginning of the prepupal period. Now it is just at 80 hours that the lymph glands show the first sign of malignant tendencies and occurrence of antagonistic (antigen-antibody) reaction. The timing of the two processes - the completion of the differentiation of the imaginal discs and the beginning of the elimination of the lymph glands as hormone-secreting organs - would therefore fit in with the hypothesis outlined above.

According to Perez (1910) on Calliphora, and Stark and Marshall (1928) on Drosophila, when the blood-forming organs release their cells at the end of the third instar these cells act as phagocytes which help in the histolysis of the larval tissues at the beginning of metamorphosis. This view cannot be confirmed from the preparations made for the present study. The released cells have nothing to do with phagocytosis, which indeed does not occur in the prepupal and early pupal development of Drosophila. Soon after the formation of puparia the cells released

from the lymph glands aggregate around the prepupal mid-gut where they eventually form the outer muscular coat of the imago. The multiplication of the spheroids which takes place at the very end of larval life thus has an important function in development, since a considerable number of these cells are required to produce sufficient muscular material.

We may now consider the conditions in the three lethals in the light of this discussion of the conditions in the wild-type.

In lethal "no-differentiation" the lethal factor seems to cause an early antigenicity of the hexagons which calls forth an antibody-antigen reaction (supplemented by the action of complement) so that the hexagons become intensively melanised. This is followed by the same consequences as we have seen in a milder form in the wild-type. That is to say, the spheroids become antigenic and thus suppressed. This leads to the cessation of the production of the differentiation hormone. The imaginal disc mesoderm released from the inhibiting action of the hormone proliferates to give place to muscle cells which break through the disc epithelium and pour into the peripodial cavity, as described in the first part of the thesis. The muscle cells as they appear in a larval substratum behave as foreign bodies and must in turn stimulate an immune reaction which brings about their pycnotic degeneration. It is noteworthy that puparium-formation occurs very late. It may be that this is directly related to the abnormal

time of the changes in the lymph glands, but it is perhaps more probable that there is some underlying process which affects both the lymph gland changes and puparium-formation. In normal development these are, of course, nearly simultaneous. According to Hadorn (1937) and many subsequent workers the ring gland secretes a hormone which brings about puparium formation. It may well be that this hormone is also concerned in the changes which normally occur in the lymph glands, but there is as yet no direct evidence of this.

Summing up, we may say that in lethal "no-differentiation" the primary lethal effect involves only the hexagons, while the subsequent disturbances are all secondary pleiotropic effects of this main primary cause.

Lethal benign.

In lethal benign, and in all stocks which show the same type of black bodies, disturbances in the lymph glands appear at roughly the time at which they also appear in the wild-type. The hexagons behave normally in so far as their proliferation is concerned. The striking feature of the abnormal phenotype is that the spheroids which in the wild-type proliferate only moderately when the glands begin to exhibit malignant tendencies, in this case multiplied to a much greater extent. The larvae can, however, carry out a successful antagonistic reaction against proliferating spheroids which become agglutinated and precipitated as black bodies as a consequence, we

suggest, of an antibody-antigen reaction. There seems, therefore, to be nothing wrong with the antibody-producing system of the larvae. The cause for the excess proliferation of the spheroids remains obscure. Presumably their multiplication is in some way controlled by genes in the wild-type and this control is lifted as a consequence of the black-body-producing mutations.

It should be pointed out that it remains unknown whether the lethal effect of genes such as lethal benign is or is not a consequence of changes brought about in the lymph glands. There are many stocks in which similar black bodies occur frequently but have hardly any lethal effect on the imagos. Further studies are being carried out to find any possible connection between the behaviour of the lymph glands in lethal benign and 1(1)7 and the occlusion of the mid-gut.

If we are correct in attributing the formation of the melanotic tumours in Drosophila to something akin to antibody-antigen reaction, then the statements by Stark and Russell, that after transplantation such tumours grow and proliferate in the host, can hardly be accepted at their face value. It is probable that any increase in size of such transplanted tumours is due to the deposition of the host-derived cells onto their surface rather than to the growth of the transplanted cells themselves.

In Drosophila, the criterion used for deciding that an agent is carcinogenic has been its action in causing the formation of the black bodies (refer to Radiation Biology by H.J.Muller 1954, ed. by A.Hollander).

Carcinogenic substances active in mammals bring about the inception of cancerous conditions in tissues which otherwise shows no signs of such tendencies. In Drosophila, the situation is very different. The black bodies themselves, as we have seen, cannot be regarded as truly neoplastic in nature, but are to be regarded as antibody-antigen agglutinates. However, the lymph glands which give rise to them do, in the normal course of events, exhibit tendencies towards proliferation and the assumption of a malignant state. The substances which have been considered as carcinogens in Drosophila act, therefore, to stimulate tendencies which in a milder degree are a normal feature of development. They should therefore be regarded as stimulants of cellular proliferation rather than as truly carcinogenic.

Lethal malignant

In "lethal malignant", larvae begin to show malignant tendencies at the normal time. The first sign of the action of the lethal factor is that these tendencies are not antagonised in the usual way. It would seem, then, that one of the main actions of the gene is to prevent the lethal carrier from producing antibodies to the malignant

and antigenic hexagons. These cells are therefore able to proliferate extensively. This is followed by the proliferation of the spheroids so that a mixed cell type carcinoma is formed. Again, there is little signs of reactions antagonistic to the spheroids. There are therefore two problems to discuss in relation to these cells. Firstly, why do they proliferate so extensively, and secondly, if the lethal factor only prevents the body from producing antibodies against the hexagons, why is antibody formation against the spheroids so weak?

As regards the first problem, it should be pointed out that in the wild-type the hexagons and the spheroids do not begin to manifest malignant tendencies at quite the same time. As illustrated in Fig. 66 the hexagons are already somewhat melanised (that is to say, have become antigenic and suffered antibody and complement reactions) at a stage when the spheroids have only just begun to undergo multiplication. Again, in "lethal no differentiation" the death of the hexagons in the early part of the third instar is followed by a considerable proliferation of the spheroids. These facts give one some grounds for suggesting that healthy spheroids possess an inherent potentiality to be malignant and that healthy hexagons exert some influence which tends to inhibit these tendencies. If now the hexagons become antigenic and irrespective of whether they become antagonised or remain unopposed, the inhibiting

activity imposed upon the spheroids is abolished, and one could account for the proliferation of these cells in the wild-type, lethal "no-differentiation", and lethal "malignant".

Turning to the second question, the apparent lack of antibody formation against the spheroids might be interpreted in two ways. We might suppose that the hexagons and the spheroids produce two different antigens and that the effect of the lethal malignant factor is to reduce the ability of the larva to produce antibodies at all. Alternatively, we may suppose that the antigens are related and that we are dealing with an overlapping reaction. It is true that in wild-type there is an antibody reaction against the hexagons at a time while the spheroids are uninhibited, but it may well be that the small number of hexagons do not produce a high enough antibody titre to agglutinate the larger body of spheroids. It is therefore not impossible to suppose that the spheroid antigen is related to that of the hexagons. Perhaps we might represent the latter as "A", and the former as "A + B". While anti-A antibody is produced against the hexagons, anti-A + B antibody is stimulated against the spheroids. Apparently the latter antibody is not produced unless the body is able to produce the former. It is significant that in Experiment 8 the injection of lymph glands from 85-hour old wild-type larvae which contained only spheroids produced an antibody reaction which affected not only

the spheroids of the host but the hexagons as well. The results of Experiment 10 in which lethal malignant larvae injected with sperm reacted antagonistically to both components of the tumour tissue, also suggest that the anti-hexagon, anti-spheroid and anti-sperm antibodies all overlap.

Nature of the malignant mutation.

One could imagine that, in the wild-type larvae, the sudden appearance of antigenic tendencies in the blood-forming organs is simultaneously accompanied by the action of a gene which stimulates the antibody-producing system of genes to respond without delay in order to give no opportunity for the hexagons to remain malignant. Alternatively, antibody production is carried out by a polygenic system with probably a major gene or genes responsible for the secretion of most of the antibody required and of whose activities the other minor partners are a function; i. e. a certain amount of antibody secreted by major components may be required in the beginning to stimulate the other genes of the system. However the explanation, the mutation under discussion could involve the evocator gene in the first postulation, or one of the major genes in the second; resulting in a comparatively inert system of antibody-producing genes.

It is noteworthy that all tumour-bearing larvae sooner or later resist their own neoplasm. Even in the

extremest cases where the imaginal discs become necrotic and the fat bodies completely infiltrated, immune reactions are in the end effectively set up. This is demonstrated by larvae which carry immovable black structures in their fat bodies, indicating that a full response to the antigenic neoplasm was organised at a very late stage after both primary and secondary growths have established themselves. On the other hand, the antagonistic response to the tumour tissue may be effective at an earlier stage before centres of secondary aggression have been formed, or when they have reached only the imaginal discs but not the fat bodies. The most usual condition, in fact, is one in which metastasis occurs but the secondary growths have not been able to make much progress. These differences in behaviour are mainly quantitative in character. They are presumably largely due to genetic differences involving genes which have a modifying effect on the major lethal mutation. However, environmental influences undoubtedly also play a part, since under unfavourable cultural conditions there is an increase in the frequency of cases with large secondary growths.

It appears then that the malignant larvae must be at first immunologically inert towards the neoplasm, but that an effective concentration of antibodies is gradually built up. It might be expected that the more inert the larva, and the slower the build-up of the antibodies, the

greater would be the concentration eventually necessary to antagonise the neoplasm which has had the opportunity to grow to a considerable size. We may therefore have appreciable quantities of antibody which yet do not reach the threshold necessary to suppress the growth of the whole neoplasm. It may be that these sub-threshold concentration of antibody play a part by inactivating the platelet cells. We have seen that these give rise to cells which owing to their local mobilisation afforded sometimes a very effective mechanism of immunity. Their anti-neoplastic role can in some ways be compared with that of the polymorphnuclear leucocytes in the blood of vertebrates which behave as phagocytes. According to Mudd, McCutcheon and Luke (1934) and others, phagocytosis proceeds more actively in immune sera. It thus appears that leucocytosis in vertebrates requires the cooperation, so to speak, of serum antibody. It is suggested that the same may be true of the platelet cell activities.

Having been led to the hypothesis that the lethal malignant factor reduces the ability of the larva to produce antibodies, we may ask which organ or organs in *Drosophila* larvae are the site of formation of these substances. There is as yet no definite answer to this problem and little guidance can be obtained by considering the evidence from vertebrates. While it is probable that

in that group antibodies, probably like other serum proteins, are formed in the reticulo-endothelial cell system (liver, bone marrow, etc.), there are also indications that they can be manufactured, at least to some extent, locally in almost any part of the body. Moreover, some authors (e.g. Carrel and Ingebrigtsen, 1912) have reported antibody formation by tissues in vitro. It may be then that in Drosophila antibodies can be produced throughout the whole body, in which case the lethal factor must be effective in every cell of the organism. If, on the other hand, antibody formation is limited to some particular tissue (as, for instance, the Malpighian tubules) then the effect of the lethal factor would be limited to these organs.

It remains to discuss the appearance of sperm in lethal -malignant larvae. In the normal course of spermatogenesis in Drosophila the testes of newly hatched larvae contain only spermatogonia. Approximately 28 hours after hatching both spermatogonia and spermatocytes occur and this remains the condition of the larval testes until shortly before pupation (Kerkis 1933, Sonnenblick 1941, Gloor 1943, Geigy and Aboim 1944). It is true that Gleichauf (1936) has reported that the testis of a fully grown larva contain stages up to and including sperm bundles. This observation has, however, usually been dismissed as aberrant.

The agglutination of injected sperm reported in

Experiment 10 indicates that the larval body can produce antibodies to sperm if they occur. It seems clear that the failure of sperm to become differentiated in the larval stages must be related to this ability of the larval body to produce antibodies against them. It seems probable that the secondary spermatocytes (which are similar to the dead sperm in showing a strong affinity for basic dyes), stimulate an immune reaction in the larval substrate and that this suffices to hold further differentiation in check. It seems likely that this may be a rather general type of reaction in embryonic development. That is to say, if an embryonic step happens, owing to some developmental abnormality, to occur earlier than ^{it} should probably do, the differentiated tissues behave as foreign bodies and bring about their own degeneration.

The bearing of Drosophila tumours on the general problems of cancer.

The most important point which emerges from the analysis of the three lethal stocks presented above is that neoplastic growth in these cases is the result of the exaggeration of tendencies which can be detected even in wild-type normal individuals. In such individuals these tendencies are held in check by antagonistic influences, but in the malignant stock these antagonistic tendencies are to a greater or lesser extent suppressed so that the

malignant tendencies can come to fuller expression. Cancer, therefore, is not something basically foreign to normality but it is a continuation and exaggeration of a normal process.

It is perhaps possible that this point of view can be applied not only on Drosophila but also to phenomena in vertebrates. For instance, Barigozzi and Dellepiane (1951) applied the test of malignancy diagnosis on 400 cases of women affected by different gynaecological conditions. This test, as reported earlier in the first part of the thesis, is based on the structural heterogeneity of malignant tissues where two types of nuclei A and B occur, both differ from each other and from normal cells in regard to their heterochromatin. In each patient the general clinical symptoms as well as the histological structure of the uterine mucosa and the cytology of the vaginal fluid were analysed. The results showed a fair agreement between the occurrence of the A and B cells and the presence of uterine cancer. There were, however, both false-positive and false-negative cases, the former being rather more frequent. In the false-positive cases and in the absence of any symptoms of neoplasm, only a small minority of A and B nuclei were found among thousands of normal elements. This was interpreted by the authors as a very early stage of cancer that might perhaps vanish later. In view of the behaviour of the Drosophila lymph gland tumours it seems possible that the small

minority of A and B nuclei found in phenotypically non-cancerous women may be a consequence of normally occurring tendencies towards uterine cancer. If this condition is assumed to appear at a definite age, then, the physiological ages of these false-positive cases should be more or less equal and should be younger than those of the positive cases.

It is, of course, not suggested that all vertebrate cancers originate in this way.

In Drosophila, the tumour is involved in an antibody-antigen reaction, the tumour cells behaving as antigen to which the individual's body reacts rather feebly. If the same principle can be applied to vertebrate cancers then we must suppose that when a malignant growth develops this indicates that the body is unable to react to its own neoplasm. The situation would then not differ from that of a person who is hereditarily unresponsive to a particular bacterial antigen and eventually dies of the disease. The main difference is that the cancer antigen is endogenous in origin while that of other diseases is exogenous. It is therefore possible that some cancers at least are like other diseases in which antibodies, if produced, are protective and play an important part in natural defence.

The results of Experiment 5, in which malignant larvae were able to overcome their neoplasm by inoculation with a further dose of the neoplasm, appears at first sight in contradiction to the genetic theory of tumour

transplantation which has been put forward on the basis of the work on vertebrate tumours. In vertebrates, if an individual can support the growth of a particular tumour there is no evidence that inoculation with a further dose of the same tumour causes an increase in the host's resistance. This difference in behaviour is to be attributed to the fact that the Drosophila larva, even without any treatment, possesses well marked powers of antagonising its neoplasm, whereas in vertebrates such antagonistic powers are totally absent. Thus in Drosophila the inoculated dose can accelerate the rate of antibody production while in mammals there are no such opposing tendencies which can be stimulated in this way.

The difference between Drosophila and vertebrates in the ability of the individual to produce immune bodies against its own neoplasm should perhaps be attributed neither to the wide gap between the two organisms nor to the lack of homology between the tumours in the two cases. It must be remembered that the Drosophila tumours studied had been produced by simple gene mutations. Many vertebrate tumours undoubtedly have a multi-factorial basis, as have mammary tumours of mice. The tendencies antagonistic to the growth of the Drosophila tumours are presumably related to the rest of the unmutated genotype; the antibody estimate of which may reach in a longer period a threshold high enough to end the malignant condition. If one could find several factors similar to lethal malignant and

synthesised genotypes containing a number of them, one would expect their action to be to some extent additive and that the larvae carrying such factor complexes would be entirely unable to antagonise neoplastic growth. They would then behave similarly to the tumour-bearing vertebrates.

If we extend our findings regarding the nature of the causation of tumour growth to represent, for instance, mammary tumours in mice, it follows that: 1) anti-neoplasm antibodies are natural immune bodies in the sera of mice, 2) low mammary tumour strains could fit genetically with the situation reviewed in lethal malignant larvae. This means that an insufficient number of malignant mutations occur in their genotypes, the malignant effect of which is antagonised by the rest of the unmutated complex. According to this postulation, non-tumorous mice of low mammary tumour strains develop the neoplasm but, before it becomes macroscopic, it is antagonised in a way similar to that of Drosophila lymph gland tumours resulting in a high concentration of anti-neoplasm antibodies in their sera. 3) High mammary tumour strains of mice are apparently deficient in these natural antibodies.

On the basis of this hypothetical approach, the possible nature of the "milk agent" in mice can be discussed

It has been established that the occurrence of mammary cancer in mice is largely dependent upon an influence which is transmitted in the milk of the mother. High tumour strain A females taken from their mothers at an early stage and nursed upon low tumour strain CBA foster-mothers gave a low tumour incidence than that expected from the strain A (Bittner 1939 and others). Similarly, when females of a high tumour strain are used as foster-mothers for young of a strain with low incidence, then the tumour frequency is increased.

The agent has been demonstrated in various organs and tissues, and it is usually suggested that it is a virus-like body, probably a ribose nucleic acid complex and a molecular weight of some 3,000,000 to 5,000,000 has been suggested.

The occurrence of a factor of some sort in the milk which adds to the susceptibility to mammary cancer, may attract attention to the importance of the milk and colostrum in the phenomenon of passive transfer of immunity. In mice, immunity is transferred to the young both before and after birth, though the latter is the more effective (Ehrlich 1892). Such a mechanism is important since young mice are incapable of producing antibodies for some period after birth. By the end of the lactation period the young become passively immunised, acquiring probably a higher antibody titre than that in their mother's sera.

Fostering susceptible young upon low tumour strain mothers involves the passive transfer of the assumed natural antibodies to the young and may confer upon them a considerable degree of immunity. Since the genotypes of high strain females are unable to synthesise these antibodies (as shown by transplantation results), the transferred antibodies do not produce their effect by passive sensitisation but by direct passive immunisation. The absorbed antibodies may be sufficient to antagonise the neoplasm at its early stage of development.

This suggests that the milk agent might be the lack of anti-neoplasm antibodies in the sera of the malignant mothers and their occurrence in the sera of the low strain mothers. It would seem worth while to apply complement-fixation reaction in search for such antibodies both in the sera of non-tumourous and low tumour strain mothers.

SUMMARY

1. Lymph glands from lethal malignant larvae were injected into the body cavity of wild-type and lethal non-differentiation larvae. The injected cells later appeared as clumps of melanised and degenerating tissue, identical with the black bodies usually referred to as *Drosophila* tumours.
2. It is argued that the formation of these black bodies should be regarded as an antibody-antigen reaction, the injected neoplasm having acted as an antigen and stimulated an immune reaction involving agglutination and spindle cell formation.
3. When a single injection of neoplastic material is made, agglutination and spindle cell formation are followed by both lysis and melanin deposition. If a second injection is made after this process is completed, the second dose of neoplasm shows agglutination and spindle cell formation, but lysis and melanin deposition do not occur. It is suggested that the two latter processes are produced by something akin to complement, which is removed from the haemolymph by the first dose.
4. When mild doses of neoplasm are injected into young lethal malignant larvae the development of the host's neoplasm is slowed down. The injected neoplasm appears, then, to accelerate the production by the host of antibodies against its own neoplasm.
5. If lymph glands from 80-hour wild-type larvae are injected into early third instar larvae they are thrown down as black bodies. The lymph glands of larvae of this

age also show black spotting when treated with hot fixatives, a reaction which, as we have seen in Part 1, is characteristic of proliferating neoplastic tissue. Some of the other cytological symptoms of the neoplastic state (e.g. the melanisation of the hexagons and proliferation of the platelets and spheroids) can also be seen in such larvae. This condition persists for some hours but gradually declines and by the 90-hour stage the symptoms of incipient malignancy have disappeared from the lymph glands. It is suggested that these changes indicate a development of malignant tendencies in the wild-type lymph glands and the inhibition of these tendencies by an immune reaction which they stimulate in the body of the animal which carries them.

6. The haemolymph from late third instar wild-type larvae was injected into the body cavity of early third instar malignant larvae. The development of the neoplasm in the host larvae was inhibited and these may be taken as evidence for the existence of anti-neoplasm antibodies in natural haemolymphs.

7. If sperm from adult individuals is injected into the body cavity of a larva the sperm become melanised and agglutinated. It is suggested that if sperm should appear in the testes of a larva they would behave as antigens and stimulate the production of antibodies which would cause their melanisation. This appears to be the explanation of the phenomena described earlier in lethal-malignant.

8. The possibility that cells released from the lymph glands in normal larvae may act as phagocytes during metamorphosis has been investigated. It is concluded that no phagocytosis occurs during the metamorphosis of *Drosophila*. The so-called phagocytes actually come together to form the muscular coat of the mid-gut of the imago.

9. The mode of action of the three lethal factors is discussed in terms of the antibody-antigen system which has been postulated. It is suggested that in lethal non-differentiation the genetic factor causes an early antigenicity of the hexagons, which leads to early suppression of them and of the spheroids, and thus to a disturbance in the production of the differentiation hormone. In lethal benign the main effect seems to be an increase in the proliferative tendency of the spheroids. This gives rise to a large number of cells, which, however, the antibody-producing system is able to immobilise in the form of black bodies. In lethal malignant the phenotype seems to be due to a reduction in the capacity to produce antibodies which are capable of arresting the normal neoplastic tendencies of the lymph glands.

10. In the discussion the bearing of these facts on the problems of cancer in mammals is briefly touched on, with particular reference to human uterine cancer and breast cancer in mice. It is suggested that general importance may attach to the conception that in normal individuals there are tendencies towards neoplastic growth which are held in check by opposing tendencies,

and that genetic factors can operate to disturb this balance by affecting either one or other of the two components.

BULSTON

EXTRA STRONG

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