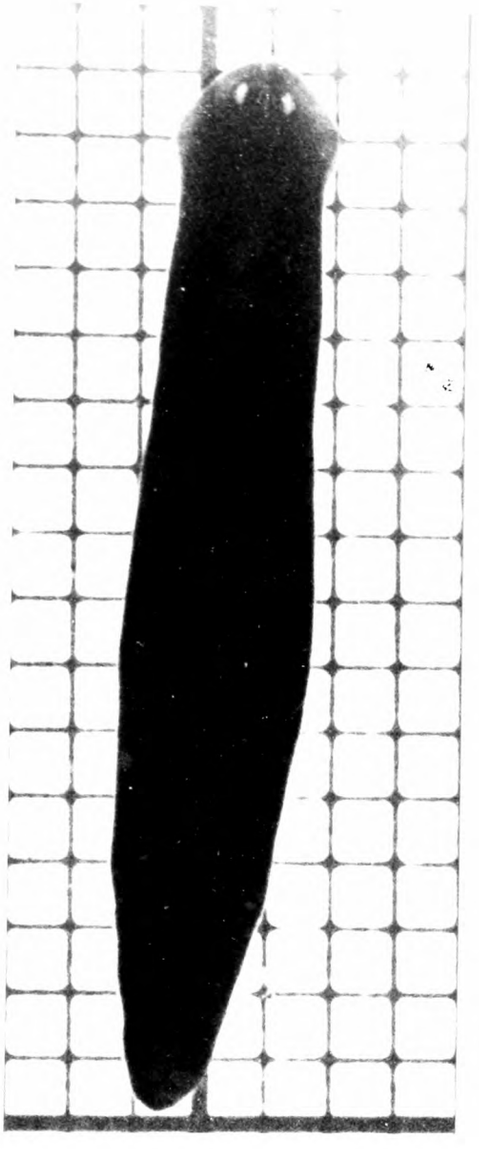

a



b

Frontispiece:

(a) The Planarian Dugesia lugubris.

(b) Neoblasts in the Parenchyma of D. etrusca.

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THE MECHANISM OF THE RADIATION EFFECT AT THE CELLULAR LEVEL

A Radiobiological Study of the Planarian Neoblast

by

C. S. LANGE

Exeter College

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Abstract

Abstract

The effect of radiation on planarians, triclad platyhelminths known for their extensive powers of regeneration, has been studied with particular reference to the cellular basis of animal mortality probability. By reference to the literature we have shown that the planarian is an organism consisting of two cell populations: nonreplicating differentiated tissues interspersed with a population of morphologically identifiable stem cells. Postirradiation survival of the organism has been explained in terms of the rate of decay of function of the differentiated tissues and the reproductive integrity of the stem cell population.

Factors known to influence planarian regenerative ability have been examined for their influence on mortality robability.

Under standard conditions pre- and postirradiation, the change in per cent survivors as a function of radiation dose was taken as a measure of the reproductive integrity of the planarian stem cells. The survival of neoblast (stem cell) reproductive integrity for D. lugubris has been determined assuming a simple relationship between stem cell reproductive integrity and animal mortality probability (q.v. chapter VI),

which necessitated the measurement of stem cell population size and repopulation probability (chapters VII and VIII respectively). This model represents the first successful quantitative description of animal mortality probability in terms of cellular reproductive integrity.

Histological studies were made to determine the number and distribution of neoblasts per animal in diploid, triploid and tetraploid D. lugubris (chapter VII). The number of neoblasts per animal (N_o) was found to be independent of ploidy. A highly significant correlation was found between the logarithm of N and the logarithm of the animal's length or volume. Other correlations revealed that older animals have a lower density of neoblasts although more of them - a point of possible relevance to the process(es) of ageing in planarians. The observed patterns of change in neoblast distribution with age suggest different modes of growth in the diploid and triploid.

The effect of radiation on head regeneration indicates that some planarians possess sufficient neoblasts to regenerate a head from radiation-sterilized neoblasts (chapter XII). The process of spontaneous fission in a fissiparous species was shown not to be inhibited by radiation.

The rate of neoblast disappearance postirradiation was studied (chapter XI) histologically in D. lugubris. The rate

of disappearance was shown to increase with dose, but not sufficiently rapidly to explain the rapid loss of ability to regenerate a head from the sterile neoblasts present unless neoblast postirradiation differentiation ability decays rapidly (hours).

Grafting and shielding experiments (chapter VIII) were done on D. lugubris to determine the number of neoblasts necessary for a 50% probability of survival, the reciprocal of this number, α_{50}, being taken as the repopulation probability for a single neoblast. The product $N_o \alpha_{50}$ is a measure of the number of neoblasts necessary to ensure survival of the planarian. On the basis of the value of $N_o \alpha_{50}$ obtained for D. lugubris we must conclude that this species does not possess a superfluity of stem cells.

An experiment designed to test the model which describes the relationship between planarian and neoblast survival has borne out a prediction of it. Thus in this system the variables considered, are sufficient to allow a definition of the survival of the organism in terms of the reproductive integrity of its stem cells. However, it is important to note that under other conditions the rate of decay of function of the differentiated tissues must also be taken into account.

Previous studies of the survival of reproductive integrity in organized tissues have dealt with a cell population

either in a state of constant turnover (under the conditions of assay) and/or where the second parameter (the extrapolation number) of the cell survival curve was not defined. This study has provided a complete determination of the dose response curve of animal cells under conditions of normal growth regulation in an organized tissue *in vivo*. Furthermore, we have been able to show that the use of the multi-target type survival curve gives meaningful results for cells under normal *in vivo* conditions, where cells are not in a state of continuous turnover.

The parameters of the diploid neoblast[1] reproductive integrity survival curve were deduced to be $D_o = 727 \pm 159$ rad and $E = 3.1 \pm 2.3$ ($\pm$ S.E.). Neither the slopes nor the extrapolation numbers of the triploid and tetraploid curves were significantly different from those of the diploids, but the polyploid values suggest that polyploids are more radiosensitive than diploids. No protective effect of polyploidy was found. The problem of polyploidy in relation to radiosensitivity is discussed.

---

1. For *D. lugubris*.

Abbreviations

Abbreviations

time	=	t
month	=	mo.
week	=	wk
day	=	d
hour	=	h or hr.
minute	=	min.
second	=	sec.
kilogramme	=	kg.
gramme	=	g.
milligramme	=	mg
microgramme	=	μg
volume	=	V
litre	=	l
millilitre	=	ml
meter	=	m
centimeter	=	cm
millimeter	=	mm
micron	=	μ
outer diameter	=	O.D.
inner diameter	=	I.D.
LMW	=	low molecular weight
MW		" "
M	=	molar
DNA	=	desoxyribose nucleic acid
RNA	=	ribose nucleic acid
number of	=	No. or #
change in	=	Δ

Chapter I

Introduction

A Modes of Approach to the Study of Radiobiology.

The fundamental tenet of radiation biology is "that the biological effects of ionizing radiations are due in some way to the chemical changes induced by the radiations" (Lea, 1955). It is the task of the radiation biologist to elucidate the nature of the "some way" both for its own sake and so as to obtain a better understanding of the unperturbed (unirradiated) system.

When a multicellular organism is irradiated it is generally assumed (e.g. Bond et al. 1965) that the organismal response (e.g. mortality) is eventually to be understood in terms of loss of function and/or viability of the component cells. The observed response (loss of reproductive integrity) of the component cells is itself thought to be explicable in terms of the integrity of the hereditary material (i.e. DNA). (e.g. Puck and Marcus, 1956). Thus to understand organismal effects we must keep an eye to the cellular level; and to understand cellular effects we must keep an eye to the cells' constituent parts.

This introductory chapter is divided into two main parts, each of which is further subdivided. In the first part we shall consider (1) models used to describe and interpret quantal post irradiation survival data, (2) models which attempt to explain organismal radiation lethality in terms of the cellular kinetics of maintenance and renewal of the organ systems

essential for life, and finally, (3) the role of nuclear factors in the determination of cellular radiosensitivity. In the second part we shall examine the suitability of the planarian as an experimental system for the investigation of cell: organism and nucleus:cell relationships, so as to throw some light on the mechanism of the radiation effect at the cellular level.

1 Inactivation Curves, Survival Curves, and their Implications

a Target Theory

Both for historical reasons, and because of the importance of methods of data analysis, we shall begin with an examination of target theory. Target theory is a collection of mathematical models for the analysis of radiation effects on single cells or molecules, all of which have in common the assumption that the irradiated material contains a number of identical independent components. Each component is held to be capable of a specific function which can be assayed quantitatively. The simplest of these models is that which generates exponential survival curves.

i Exponential Survival Curves

If a single component in each unit must be intact for that unit to function, then for a given dose, D, and a sensitive volume, V, the average number of such events per target will be VD; where D is in units of randomly distributed

inactivating events per unit volume, uniform throughout the irradiated material, and V is the volume in which an event must occur for a single component to be inactivated. Because the inactivating events are randomly distributed, the Poisson formula can be applied to determine the fraction of targets (components), F, to receive a certain number of events, n:

$$F = \frac{e^{-a} a^{n}}{n!} \qquad \text{Eq. IA-1}$$

where, a=VD=average number of events per target

and, n=number of events.

Thus, if one or more events will inactivate the unit, then the surviving fraction is that F, for which n=0:

$$F_s = e^{-VD} \qquad \text{Eq. IA-2}$$

i.e. an exponential survival function. This is called a "one-hit" curve.

The "sensitive volume" (= target size) can be calculated if it is known that the inactivation is caused directly by the ionization and that mediation by the milieu (indirect effect) can be excluded. For an average of one event per target, VD=1, (survival = $e^{-VD} = e^{-1} \sim 0.37$) a dose $D_o = \frac{1}{V}$ rad will produce the following number of ion clusters per cubic cm:

$$D_o \times 100 \times \frac{1}{1.60\times10^{-12}} \times \left(\frac{1}{75}\right) \times \rho = 0.83\times10^{12}\rho D_o$$

$$\frac{/\text{rad}/}{} \quad \frac{/\text{erg}/}{/\text{gm}/\,/\text{rad}/} \quad \frac{/\text{eV}/}{/\text{erg}/} \quad \left(\frac{/\text{ion cluster}/}{/\text{ev}/}\right) \frac{/\text{gm}/}{/\text{cm}^3/} \quad \frac{/\text{ion clusters}/}{/\text{cm}^3/},$$

where the target is large relative to the dimensions of an ion cluster, and the molecular weight of the target can be calculated to be:

$$\text{M.Wt.} = V\rho \times 6.02 \times 10^{23} = \frac{6.02 \times 10^{23}}{0.83 \times 10^{12} D_o} = \frac{0.72 \times 10^{12}}{D_o}$$

Eq. IA-3

If the inactivation is due to a single ion or to the primary ionization, the $\left(\frac{\text{ion cluster}}{75\text{eV}}\right)$ term is replaced by the appropriate inactivator unit divided by its energy of production.

For densely ionizing radiation, there may be much "wastage" due to overlapping of ionizations. To allow for this, Lea (1955) introduced his associated volume calculation, i.e. the volume associated with each ion cluster such that any target, the centre of which lies within this volume, will be inactivated. The degree of overlap of these volumes is a measure of the wastage.

For very densely ionizing radiation, inactivation could be said to occur if such a particle passes through the target. Thus if dose flux is given as B particles per unit area and

the mean target cross section is S, the mean number of tracks crossing a target is SB, and by Poisson's formula the proportion of targets not traversed (not hit) = e^{-SB}. By calculating V for sparsely ionizing radiations, and S for very densely ionizing radiations, one can obtain, $\frac{V}{S}$, the mean target thickness, and thus make deductions as to the shape of the target.

Zirkle and Tobias (1953) calculated an effective inactivation volume allowing for diffusion of the energy from the site of the primary event. However, as long as the medium is isotropic, the mediation of diffusing radicals will not change the shape of the curve.

ii Nonexponential Survival Curves

Target theory can generate two single component nonexponential survival curves and a multitude of multicomponent ones.

The former category consists of the family of multihit survival curves which can be interpreted either on the multitarget model (each unit has n targets and all n must be hit for an inactivation to occur), or on the multihit target model (each unit has one target but n hits in that target are necessary for inactivation).

aa. For the multitarget model, the probability of a single target surviving is, as above, e^{-VD}. The probability of its inactivation is $1-e^{-VD}$, so that the probability of all n targets in a single unit being hit is $(1-e^{-VD})^n$. Thus the probability of survival, S, is:

$$S = 1-(1-e^{-VD})^n \qquad \text{Eq. IA-4}$$

which for large D reduces to an exponential and can be expressed as

$$\ln(S) = \ln(n)-VD \qquad \text{Eq. IA-5}$$

V is then equal to the reciprocal of the D_o (37% dose-slope) of this exponential, which when extrapolated back, will intercept the ordinate at ln(n).

bb. The multihit target model survival curve can be calculated directly from the Poisson formula as the sum of the proportions of all those targets which have sustained n-1 or fewer hits, i.e.

$$S = e^{-VD} \sum_{i=o}^{n-1} \frac{(VD)^i}{i!} \qquad \text{Eq. IA-6}$$

This survival curve, even at high doses does not reduce to an exponential but continues to curve on a log-linear plot.

cc. The simplest of the multicomponent nonexponential survival curves is the type which can be expressed as the sum of two or more exponentials, the slopes being interpreted as the target sizes, and the extrapolated ordinate intercepts as the incidence of each target size in the original population. The survival equation of such populations can be written as

$$S = 1-(ae^{-V_1D} + be^{V_2D} + \ldots + ie^{-VjD}) \qquad \text{Eq. IA-7}$$

dd. A slightly more complex multicomponent curve, which has been shown to be worth consideration (Bender and Gooch,

1962; Porter, 1964), is the product of an exponential component and a multitarget component. This model provides a curve which starts with an initial negative slope (the multitarget curve starts with zero slope) and eventually becomes exponential (the multihit target curve continues to curve). This can be written as

$$S = e^{-UD}\left\{1-(1-e^{-VD})^{n}\right\}, \qquad \text{Eq. IA-8}$$

where U is the $1/D_o$ for the one hit component, and V and n are as in Eq. IA-4. For large D, this reduces to the exponential:

$$S \sim ne^{-VD} \cdot e^{-UD} = ne^{-(U+V)D}. \qquad \text{Eq. IA-9}$$

Another two component model, where one of the components is a double exponential has been suggested by Haynes (1964). According to this model:

$$S = e^{-kD + \alpha(1-e^{-\beta D})}, \qquad \text{Eq. IA-10}$$

where, k is the inactivation probability per event of the target (V),

β is the rate of inactivation (inhibition) of the proposed reactivation (recovery) mechanism,

and α is the contribution of the reactivation mechanism to the back extrapolation of the log assymptote (αk) on the dose axis. When plotted semilogarithmically, this function assymptotically approaches a limiting slope of $-k$ and has an initial

slope at zero dose of $-(k-\alpha\beta)$. It reduces to a simple exponential in two cases:

(1) when $\beta = 0$, i.e. there is no recovery mechanism, and

(2) when $\beta D << /$ (low doses), in which case the exponential constant reduces to $(k-\alpha\beta)$. Thus the initial slope refers to cells with a fully functional recovery mechanism and the final slope to cells with a fully inhibited recovery mechanism.

iii Successes and Failures

Target theory has been of considerable significance when applied to the study of molecules irradiated in the dry state. A graph (Hutchinson and Pollard, 1961) of the correlation between target theory-predicted molecular weight (radiation molecular weight) and physicochemically determined molecular weight (physical molecular weight) shows a very close scatter of points about the line of 1:1 correlation for over four orders of magnitude. Several points, however, lie considerably further from the line than their experimental errors alone would warrant. Nevertheless, few points deviate from the line by more than a factor of 2 to 3. Thus, with few exceptions, the mechanisms of inactivation for enzymes, nucleic acids and some viruses in the dry state are consistent with target theory.

When TMV, a long rod-shaped virus, were aligned in a known direction and irradiated with the major axis at an angle α to the deuteron beam, the calculated radiation cross section

varied as sineα (Pollard and Whitmore, 1955). When the beam was held perpendicular to the viral long axis, rotation in that plane had no effect, the radiation cross section (comparable to the physical size of the virus) remaining maximal. When cells were irradiated, the enzyme inactivation curves for acetylcholinasterase and Co-A were the same as for in vitro irradiation of the dried enzymes (Serlin and Cotzias, 1957; Hutchinson and Norcross, 1960.). Hutchinson and Pollard (1961) also cite several examples of identical radiation inactivation curves for enzymes in dried cells and for dried extracts. Ginoza and Norman (1957) found that the target for inactivation of TMV was the same for the infective fraction alone as for the complete virus. The radiation molecular weight is in good agreement with the physical molecular weight of the TMV RNA molecule.

The above successes are all for dry materials. All of the survival equations and/or models are reduced to mere curve generators if the milieu (medium) mediates the inactivation process(es). The molecular weight and target size calculations are valid only if the radiation damage is produced directly by radiation (direct effect). If the medium intervenes, then target sizes, etc. have no meaning as the nature of the chemical reactions of the radicals can have more influence than the nature of the target (Bacq and Alexander, 1961).

Where the radiation process is indirect, the shape of the

dose-response (yield) curve depends on the nature of the reaction product. If the product does not react with the free radicals created in the medium by the radiation, then the relationship will be directly proportional to dose (i.e. linear). If the reaction product does react with free radicals in the same way as the original molecule, then it will act as a protector in that a dose sufficient to produce an average of one radical per target will, because of the random behaviour of those radicals, leave 37% of the targets untouched; the radicals meant for them having reacted with already inactivated molecules.

Where random inactivation is the mechanism, the shape of the dose-response curve must be semi-logarithmic relationship exponential giving a linear and definable by a slope (D_o or D_{37}) and an intercept (n or E). Thus except for the indirect effect non-reacting product, which is unlikely to be of importance in biological systems (as only very small molecules are likely to become unreactive towards free radicals after one reaction), the shape of the dose-response curve tells us nothing about whether the mechanism of inactivation is direct or indirect (Bacq and Alexander, 1961). When inactivation is less than 25%, the exponential curve cannot be distinguished from a straight line, so that in this region one may properly speak of a linear dose-effect relationship.

Alper (1961) reviews the successes of target theory as applied to viruses, phage, transforming principle, and single

cell organisms such as bacteria, amoebae and paramecia. Its application to the former three appears successful. For the single cell organisms however, the contradictions (or complications) are such that the "hit number" (the extrapolated intercept of the exponential portion of the multitarget curve) had to be renamed simply the "extrapolation number" (Alper, Gilles and Elkind, 1960). Alper also discusses the many external factors which affect the shape and position of the survival curves obtained by Puck and Marcus (1956) and Hewitt and Wilson (1959), for asynchronous exponentially growing mammalian cells (the former in vitro, the latter in vivo). Although cells in all stages of cell cycle are in the irradiated population, and although from studies of the radiosensitivity of synchronous populations of germ cells or zygotes it was known that the various stages of the cell cycle differ in their sensitivities, the survival curve shows no signs of a more resistant fraction. This observation led Alper (1961) to conclude that the radiosensitivity of cells remains constant throughout the division cycle. We now know from studies on synchronous cell populations in vitro that this is not the case (Terasima and Tolmach, 1961). The asynchronous survival curve is made up of the sum of the survival curves for each phase of the cell cycle.

Bacq and Alexander (1961) point out that even where applicable (i.e. in the case of molecules) target theory can give no

more than an order of magnitude for target dimensions for the following reasons:

(1) the energy to produce a primary ionization (or the number of ionizations produced by a given dose) in a solid is only a guess (to within a factor of 4),

(2) added substances can substantially alter the inactivation dose. Thus the apparent target size is very much a function of irradiation conditions, and unless the effects of all other substances in an impure preparation are known, radiation methods cannot be used to obtain accurate dimensions of biologically-active molecules in the impure state. If these limitations apply at the molecular level to the solid state, how much more so do they apply to macromolecules in vivo!

The concept of a D_o has no meaning when dealing with complex systems such as mammalian whole-body irradiation. At the molecular level, energy is randomly distributed between the molecules and thus accounts for the survival of molecules, whereas the 37% survival dose for animals leaves an equal amount of energy in each animal, and survival is a matter of biological variation or local micro variations in dose distribution (Bacq and Alexander, 1961). If all animals at risk were identical in their radiosensitivity, the dose response curve would approach a step function - all or none survival. The heterogeneity of the population at risk tilts and curves the dose-response function away from a step function into the

characteristic shape of the sigmoid curve. The nature and properties of this dose-effect relationship are discussed in Chapter IA 1c.

Having derived several formulae to generate curves of the general shape of the dose-response data observed, one might wish to attempt to fit these curves to the data and determine which curve, and hence which model, fits best.

The simple exponential is of use both at the molecular level and in some cases at the cellular level. Many bacteria show an exponential X-ray survival. Révész and Littbrand (1964), generalizing from their observations on a mammalian ascites tumour line, suggest that under sufficient anoxia (< 3 pp million O_2 in gas phase) all X-ray cell survival curves are simple exponentials. Recently, Hall and Cavanagh (1967) presented evidence from Vicia faba in support of this suggestion. Neutron and other densely ionizing radiations also produce exponential cell survival curves (e.g. Deering and Rice, 1962).

The multitarget model has been, by far, the most popular X and γ-ray survival curve for mammalian cells in vitro and in vivo; the radiosensitivity of almost all cell lines being quoted in terms of the D_o's and extrapolation numbers of this type of curve.

The multihit target model is just as readily fitted to the cell survival data as is the multitarget model. A judic-

ious choice of constants gives curves indistinguishable from multitarget type curves for more than seven orders of magnitude in survival. (Porter, 1967)

The multicomponent survival curve of Eq. IA-8, has been shown to give a better fit (significantly smaller residual χ^2) to some of the survival curves based on the in vitro clonal technique (Puck and Marcus, 1956), e.g. for L strain mouse fibroblasts (Porter, 1964), and Girardi heart and Hela S-3 cells (Bender and Gooch, 1962, for the latter two cell lines), as compared to the multitarget model (which the data also fits, but not as well). Oliver (1964) also uses this multicomponent model for the survival of Vicia faba meristem cells.

It should be noted that the data to which the above curves are fitted are based on three different methods of determining cell survival:

(1) clonal technique in vitro (Puck and Marcus, 1956),

(2) back extrapolation of growth curves in vitro (e.g. Alexander and Mikulski, 1961) and in vivo (e.g. Révész and Littbrand, 1964),

(3) dilution technique in vivo (e.g. Hewitt and Wilson, 1959). For the first, survival of reproductive integrity has been defined (Puck and Marcus, 1956) as the ability to form a colony of 50 cells or more within a specified number of days (<20). Nias, Gilbert, Lajtha and Lange (1964) and Sinclair

and Morton (1963) have shown that some of the "non-surviving" colonies do in fact survive but merely grow at a much reduced growth rate. In the latter two methods, the slow growth of the "non-surviving" or "small colony" cells will produce, at most, a perturbation in the growth curves within the time limits of the experiment. However, the ability of such cells to continue to grow is significant, both in terms of the assumptions of the curve generating models and for long term predictions as to the behaviour of such populations. For instance, a tumour "cured" by the definitions of the clonal technique would reappear at a later date, growing more slowly than before. This phenomenon is not unknown to radiotherapists.

Thus we see that the estimation of cell survival is complex. The multiplicity of models which can be fitted to the data make it quite clear that we can not look to the models for mechanisms, but must consider them merely as a useful shorthand with which to describe the data.

b Poison Theory

The possibility that the effects of radiation could be due to the production of a stable poison(s) is generally dismissed and relatively little work has been done in this direction. The poison need not be very stable if it is produced in situ and consequently has a fair chance of reaching a biologically important molecule. If the poison were produced in situ, then sigmoid survival curves would follow; but then the

exponential curves frequently observed with ionizing radiation could only be interpreted by assuming a most unusual distribution of resistance. However, the killing of bacteria by extracellular poisons such as phenol often closely resembles an exponential curve, yet here there can be no question of inactivation of a single site (Bacq and Alexander, 1961).

In a symposium on the implications of organic peroxides in radiobiology (ed. Feinstein, 1963) the general consensus was that peroxides have been detected in irradiated tissue, that the indirect effect (dry vs. wet, in vitro and in vivo, Hutchinson cit. Guild) is responsible for two thirds of the damage to DNA in a wet cell, the other third being due to direct effect; and that radiation doses of the order necessary for cellular loss of reproductive integrity[1] would also produce sufficient peroxide radicals to alter one pyrimidine base in a thousand. Horgan and Philpot et al. (1957) found that organic peroxides of the type which can be detected after whole body irradiation are extremely toxic and the amount needed to kill is of the same order as that produced by a lethal dose of X-rays.

For the poison theory to work, peroxides cannot be the

---

1. Latarjet noted that 1000r to mammalian cells in vitro yields 10^{-3} survival, yet this dose is sufficient to alter 10^{-3} pyrimidine bases. A similar degree of substitution by bromouracil (Budr) or fluorouracil (Fudr) is extremely damaging to a cell and such a degree of pyrimidine base alteration is of the right order for inactivation of transforming principle.

only poisons because peroxide yield falls with rising L.E.T. while R.B.E. rises (Bacq and Alexander, 1961). However, a poison with a molecular weight of 1000 produced in situ with an ionic yield of unity would not have to be more toxic than strychnine (500 r would yield 50 mg. in man); so quantitatively the poison theory is not absurd.

The following observations were put forward by Bacq and Alexander (1961) as failures of the poison theory:

(1) symptoms are different for peroxide death,

(2) much larger radiation doses are needed when only the cytoplasm is being irradiated, thus why is the cell not poisoned by peroxides in the cytoplasm?

(3) non-dividing differentiated cells should be poisoned as well as dividing cells but this is not observed. These arguments are easily answered. The in situ production of peroxide would probably yield totally different concentrations and distributions of these molecules than could be expected to obtain after injection of peroxide into a rat or mouse, or even a cell; peroxide produced in the cytoplasm may not be able (or as easily able) to reach the more sensitive sites in the nucleus (as they admit), and of course sufficiently high doses of irradiation to the cytoplasm will kill a cell (Zirkle, 1957). As to dividing versus non-dividing cells, Latarjet's observations (q.v. footnote p.16) as to the effects of low

concentrations of Budr or Fudr, also point out that the cell may not show any loss of function until called upon to divide. Thus the major obstacles to the peroxide-poison theory would seem to be a lack of more direct proofs and the R.B.E. discrepancy with L.E.T.

c The Sigmoid Survival Curve

When the survival curve is sigmoid as some function of dose, then for purposes of curve fitting and statistical analysis, it is desirable to transform the variables so as to obtain a linear relationship. There are at least five such transformations:

(1) The sine curve -

$$P = \tfrac{1}{2}\left\{1 + \sin(\alpha + \beta D)\right\} \quad \text{for} \quad -\frac{\pi}{2} \leq (\alpha + \beta D) \leq +\frac{\pi}{2}$$

Eq. IA-11

This curve has the advantage of a constant weighting coefficient (Knudsen and Curtis, 1947)[1], but the disadvantage of being applicable for only a finite range of doses.

(2) The tangent curve -

$$P = \tfrac{1}{2} + \frac{1}{\pi}\arctan(\alpha + \beta D) \qquad \text{Eq. IA-12}$$

This was proposed by Urban (1910)[1] and extended by Finney (1947, 1949)[1], but is not commonly used.

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1. Cited in Finney (1962).

(3) The logistic curve -

$$P = 1 \Big/ \left\{1 + e^{-(\alpha + \beta D)}\right\} \qquad \text{Eq. IA-13}$$

This curve, for which Berkson (1944, 1946, 1949)[1] defined the logit, analogous to the probit (see below), and developed a minimum χ^2 method for the fitting of the logit regression line, is used occasionally.

(4) The probit curve -

$$P = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{Y} e^{-\frac{1}{2}u^2}\, du \qquad \text{Eq. IA-14}$$

where $u = \left(\frac{D-\mu}{\sigma}\right)$,

μ is the mean and σ is the standard deviation of that mean for the response.

The probit curve was in essence first suggested by Gaddum (1933)[1] as the normal equivalent deviate (N.E.D.) the latter being defined as the deviate of the normal curve in units of standard deviations. This was improved by Bliss (1934)[1] who defined the probit as the N.E.D. +5 so as to eliminate negative values. Fisher (1935)[1] developed a maximum likelihood method for the fitting of the probit regression line.

(5) Multihit or multicomponent curve(s) of the forms given in Eqs. IA-4, 7, 8 can be fitted by a maximum likelihood procedure, q.v. Porter (1964).

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1. Cited in Finney (1962).

Faced with this variety of methods one may be at a loss as to which to choose, but as Finney (1962) points out:

"The logit and angle transformations seem to have less theoretical recommendation than the probit; however inexact the assumption of a normal distribution of log tolerance . . . (or tolerances) . . . may be, there is no reason to suppose that the tolerance distributions to which they correspond are any closer to the truth. Nevertheless, only very numerous data could discriminate between the three, as even the limited distribution that gives the angle transformation approximates to the normal except at extreme values. In practice, conclusions drawn from the same data by each of the three methods will often agree sufficiently for the choice to be unimportant."

Probit analysis may well be the method of choice, if only because of the lucid exposition of the procedures of this method to be found in Finney's (1962) book on this subject.

Cell survival data have usually been handled by methods relevant to the target theory curves, and do not readily fit one of the other mentioned distributions. Animal survival data could be made to fit the target theory curves (although not very well) but as the shape of the sigmoid survival curve for multicellular organisms can be taken to be a measure of the biological variation between test organisms <u>per se</u> (the relative responses of animals from inbred strains as compared with heterozygous strains supports this interpretation - Bacq and

Alexander, 1961) there has been little inclination to do so. As the sigmoid dose-response curve is characteristic of biologically variable test animals (i.e. there are uncontrolled and not understood factors in play), this latter interpretation is preferable. Thus the shape of the curve is a measure of test animal variation per se and tells one nothing about the mode of action of that which is being assayed.

In simpler systems such as single cell survival, what is the meaning of the sigmoid dose-response? Do we really observe the inactivation of molecules (as a multihit process) or do we observe the effects of unknown and thus uncontrolled factors? Should it be possible to describe quantitatively animal survival in terms of cell survival, and that in terms of molecular survival, then the answers may become a little more clear.

2 Organismal Radiation Lethality

In this section we shall consider whole-body radiation-induced lethality of multicellular organisms.

There are two basic modes of approach to the study of organismal radiation lethality. The first mode concentrates on the observable mortality, and by use of those statistical methods relevant to the sigmoid dose-response curve (probit analysis) determines the slope and intercept, LD_{50}, (with their confidence limits) of the survival curve, and the distribution of mortality times. The variation in these parameters with dose-rate, total dose, fractionation, L.E.T., etc. are all use-

ful data. Combined with anatomical studies, much can be (and has been) learned as to which organ systems are of greatest import to the observed effect.

The second mode attempts to measure cell survival in organ-systems (particularly those which play a major role in the animal postirradiation mortality syndrome) in order to understand whole-body dose-response curves. The spleen colony forming units (cells) of Till and McCulloch (1961) are perhaps the most widely studied system using this approach. Somewhat similar but not as well understood, is the erythropoietic stem cell system which has been studied by Lajtha and Suit (1955), Gurney and Mackman (1961), Gurney et al. (1962), and many others. The skin clones of Withers (1967) and later Emery (1967) are another such system. Unfortunately, neither of the haematopoietic stem cell systems (erythropoietic and C.F.U.) can as yet explain in detail the processes leading to postirradiation death of the organism. This may be, in part, due to the many variables involved. Moreover, influences of other tissues or organ systems may play a major role, e.g. haemorhage, infection, and reticuloendothelial effects [Hammond (1954), Lajtha (1966), Byron and / Byron (1967)]. The situation is truly complex.

Although many mathematical models have been reported for various special and simple cases, so far there is no model which can quantitatively explain the survival of multicellular animals in terms of their cellular radiobiology.

In their review monograph, Bond et al. (1965) ably advocate the thesis that mammalian radiation lethality is to be understood basically in terms of the cellular kinetics of maintenance and renewal of the organs and organ-systems essential for life. We shall try to extend this thesis to cover all multicellular organisms.

Let us consider the organism schematically portrayed in figure I-1. It starts with a stem cell (the zygote) which reproduces itself. In some cases, there will always be a totipotential population of stem cells (e.g. Planariidae). In other cases all of the stem cells may differentiate to form pluri - or uni - potential blast cells (e.g. blast cells of ectoderm, mesoderm, and endoderm; or the erythroblasts, myeloblasts, and megakaryoblasts of the bone marrow). In still other cases, all the blast cells (of a given line) may differentiate to produce self-replicating functional cells (e.g. liver), and in a few cases all the blast cells (of a line) may differentiate to produce non-replicating functional cells (e.g. mammalian nerve). The organism is able to survive so long as sufficient functional cells remain to carry out the vital functions of each of the essential organ systems. Thus the lower the rate of wear and tear (and thus of renewal) and the higher the excess of functional cells above the bare minimum consistent with continued function of such an organ-system,

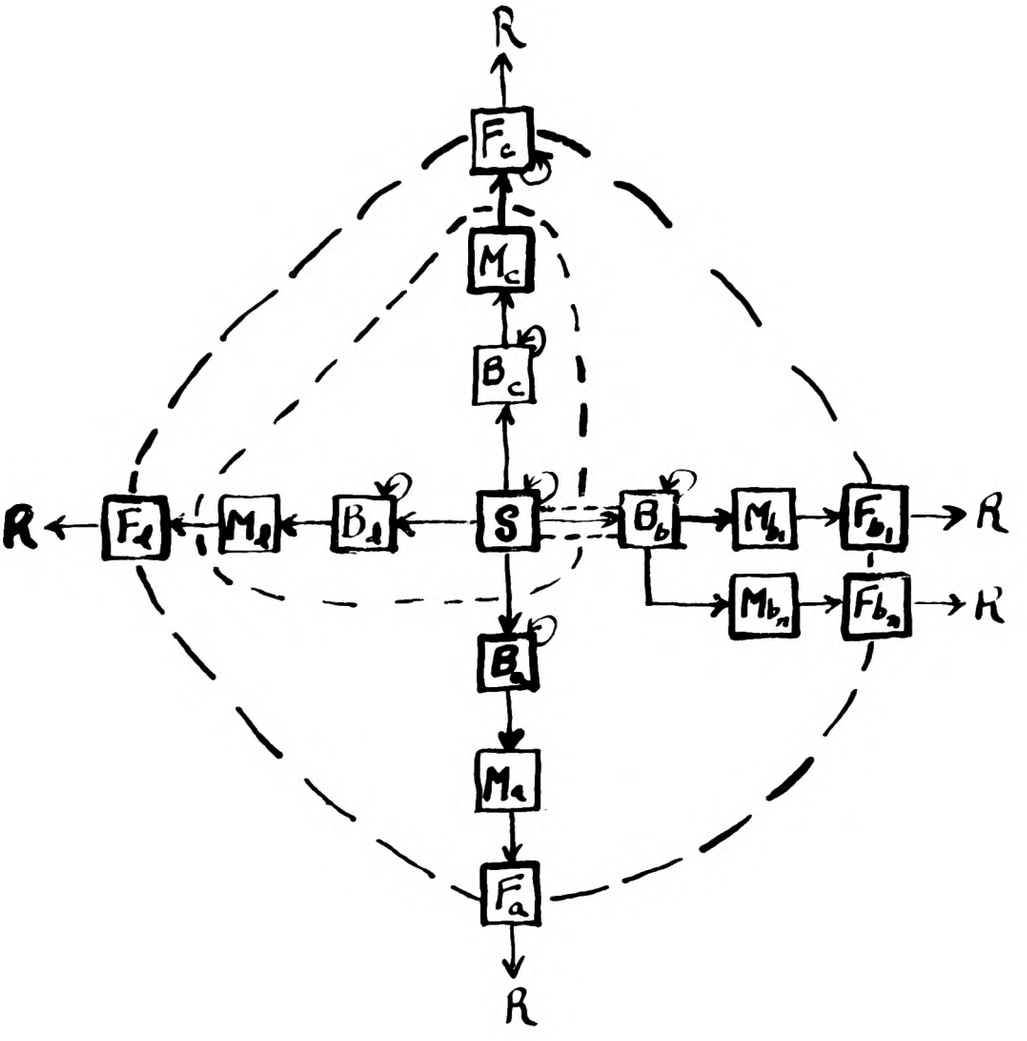

Figure I-1

Schematic Representation of an organism

The square "S" in the centre represents the stem cell compartment, which at its earliest stages consists of the zygote and then the blastomeres. The arrow leaving the square and returning represents mitotic renewal (maintenance). The arrows leaving the square represent differentiation. The inner dashed ring represents the period of embryogenesis and the outer dashed ring the organism. B = blast cells, M = maturation, F = functional tissues, R = removal of surplus or worn and damaged cells. The subscript "a" line is an example of postembryonic tissues deriving from unipotential blast cells. The "b" line represents postembryonic tissues deriving from pluripotential blast cells. The dashed corridor linking S to B_b indicates that in some cases the pluripotential blast cells may derive from postembryonic totipotential stem cells. The "c" line represents postembryonic self maintenance of a differentiated tissue by mitosis (e.g. liver) and the "d" line represents non-replicating postembryonic differentiated tissues (e.g. nerve).

the longer the latent period preceeding the expression of damage to precursor cells in the form of organ failure and organismal death. As with the weakest link of a chain, the weakest part of an essential organ or organ-system determines the time of mortality. While the organ-system continues to function, it is the degree of depopulation (in terms of loss of reproductive integrity) of the relevant precursor cells and the rate at which they repopulate their own compartments (repair), and provide new functional cells (replacement) that will determine the long term survival of the organism.

Thus the common mammalian log dose vs. log survival time triphasic curve (figure I-2) can be analysed and understood as follows:

(1) The low doses (<100r) have very little effect (< 5% life span shortening. Hursh cit. Bacq and Alexander, 1961).

(2) Between 100 and 700 r, both the bone marrow and the gut (as well as other organ systems) show extensive damage. A bimodal distribution of survival times, with 3-5 days (gut syndrome) and 10-20 days (bone marrow syndrome) as the two modes, is obtained.

(3) Between 700 and 1200 r the proportion dying at 3-5 days increases and all animals show extensive denudation of the gut epithelium. Here we see that the bone marrow stem cells are more radiosensitive than are the gut stem cells (as dose increases, cause of death shifts from bone marrow to gut syn-

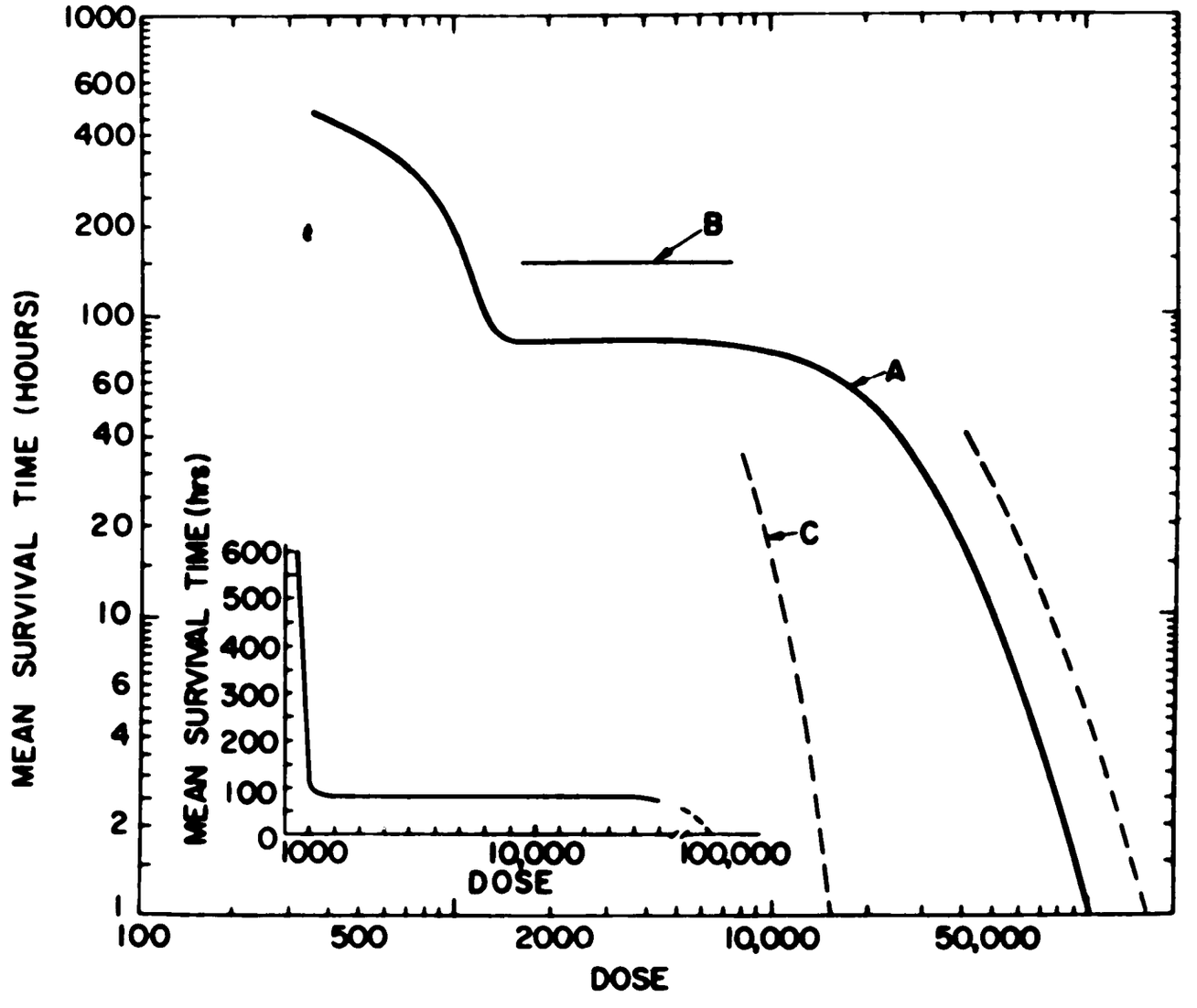

Figure I-2

Log dose vs. Log survival time /from Bond et al. (1965)/

drome) but because of the longer functional life of the differentiated products of the bone marrow, the longer the survival time and thus time for the repair of that damage for those dying of that syndrome. However, as the dose is increased (and although the bone marrow may be almost completely devoid of stem cells, the time of death of that syndrome is not brought substantially forward) the more resistant gut stem cells become depleted and as the functional cells which they produce have a lifespan of only 3-4 days, when they fail, it is the gut syndrome with its shorter survival time from which the animal succumbs.

(4) From 1,200 to 15,000 r survival time remains unchanged, i.e. there are no other vital organ-systems with a faster renewal rate and a lower precursor cell radiosensitivity than the gut, so survival time is not shortened. (We ignore here the slight life-span shortening due to increased functional cell damage resulting from higher doses.)

(5) From 15,000 to 100,000 r, survival time drops to a matter of hours. In this dose range functional cells are themselves affected, thus hastening denudation and organ failure.

(6) Above 100,000 r survival time drops to minutes. This is the dose range of 'molecular death', i.e. the basic biochemistry of the cells is thrown awry and all function rapidly ceases.

Thus we see that if one precursor cell population is more radioresistant than another and has a slower renewal rate of its functional products, then the effects of the loss of those precursor cells will be masked as the animal will die at an earlier time of another syndrome. It is for this reason that we do not observe skin, lung, bone, etc. syndromes after lethal whole-body irradiation. The effects of radiation on the replacement-cell populations of these organs can be observed, only by localized irradiations such as will not induce bone marrow or gut death. However, as Bond et al. (1965) point out, "In radiation syndromes all organs . . . become affected, and the clinical picture seen represents the synthesis of . . . (their interactions)." Moreover, mortality and its time course, for both bone marrow and gastrointestinal syndromes, have been shown to be very much dependent on the integrity of systems of defence against infection (Byron, and Lajtha, 1966).

We also note that, aside from life-span shortening by the lowest doses, mammalian radiation death occurs within 30 days of lethal exposure. Because much of the early radiobiological work was done on mammals, it was found to be convenient to take the experimental end point as 30 day survival and calculate the $LD_{50/30}$. In an attempt to compare the radiosensitivities of various lower species, several authors blindly adopted the $LD_{50/30}$ as their unit of comparison and found extremely high

degrees of radioresistance in the lower orders; e.g. it was shown by Donnelly and Jahn (1964) that for the adult salamander Desmognathus fuscus, 9 kr caused no mortality within the first 18 days postirradiation but 1 kr was 100% lethal if observations were extended over a 60 day period. Thus an end point of 15 or 30 days survival for this species would produce most misleading results.

3 Determinants of Cellular Radiosensitivity

Sparrow (1962) in a review of the role of the cell nucleus in determining radiosensitivity, reported on work performed over many years by himself and colleagues, involving acute and chronic irradiation of many plant species. These species cover a wide range of nuclear volumes and radiosensitivities. Sparrow and Miksche (1961) found a correlation between log nuclear volume and log radiosensitivity (dose rate to produce severe growth inhibition) such that plants with large nuclear volumes tended to be more radiosensitive than those with small or medium ones. For some species, a direct correlation could be shown between nuclear volume and DNA content/cell, but attempts to correlate DNA content/nucleus with radiosensitivity yielded poor results. However, if the DNA content/nucleus was divided by the chromosome number, the resultant mean DNA content/chromosome was found to correlate very well with radiosensitivity.

On the basis of micronuclei production, Sparrow and Evans (1961) concluded that the larger the chromosome or nuc-

lear volume, the more chromosome breaks occur per r of exposure. They also compared the tolerance ratios (ratio of tetraploid to diploid or octoploid to tetraploid, etc. dose rates to produce severe growth inhibition) of 8 pairs of natural polyploids and found a mean ratio of 1.67 ± 0.06 (assuming no error in estimation of tolerance doses). The slope of the regression line for each polypoid set would be a much better figure for comparison of this protective effect. Also as cycle times are not known, acute exposures could have been of greater value. If this ratio is in fact different from 2, the explanation of this may well lie in cell cycle time differences, which under conditions of chronic or fractionated irradiation have been shown to be of considerable importance (e.g. Sparrow and Evans, 1961; or Hall et al., 1966). As the polyploid is in general slower growing than the diploid, the increased dose per cell cycle would tend to decrease the degree of protection afforded by the redundancy of information.

If growth inhibition is due to deletions of pieces of chromosomes after breakage, then theoretically, Evans and Sparrow (1961) show that, an increase in the number of chromosome arms (even without polyploidy) should exert a protective effect. In support of this model, they show that Luzula, which has diffuse centromeres, is extremely radioresistant compared to Vicia or diploid Lilium, and conclude that it is the loss of genetic material rather than chromosome breakage per se

which is the critical event resulting in a cell's loss of reproductive integrity.

From the data correlating radiosensitivity and nuclear volume, Sparrow (1962) calculated the amount of energy absorbed per day per nucleus (at the exposure necessary for severe growth inhibition) to be $(67 \pm 6.4) \times 10^3$ ionizations/day or 2.2×10^6 eV/day. As DNA content per nucleus did not correlate well with radiosensitivity, whereas DNA content per chromosome did, Sparrow concluded that the determinant of biological damage is not a given proportion of DNA damage but rather a relatively constant type of event such as deletion following chromosome breakage.

Sparrow's major conclusion was that if the chromosome number remains reasonably constant, then the average number of ionizations per nucleus to yield a specific effect would change only slightly, even with a major change in nuclear volume. Or, put another way, the apparent radiosensitivity differences bet-

ween diploid species disappears if energy absorbed per nucleus is considered instead of radiation flux. In Sparrow's own words:

"The origin of this paradox is that ionizing radiation generally has been measured in units which embody a specified macroscopic volume (or weight) and, with either of these methods, no allowance is made for variation in microscopic target size (nuclear or chromosome volume). It is thus clear that the microscopic target determines what, for the individual cell, is absolutely vital, i.e. the actual absorbed energy per unit of exposure or absorbed dose."

This conclusion is carried a step further. Except in closely related species, chromosome number and nuclear volume often behave as independent variables. As correlations exist between mean DNA content per chromosome vs. radiosensitivity, and DNA content vs. nuclear volume, he calculated the mean chromosome volume (assuming that the nucleolus occupies a negligible fraction of the nucleus) and correlated this with radiosensitivity (to acute exposure). This correlation was very good. However, as chromosome number correlates poorly with nuclear volume, the two variables are independent and both must be considered when "evaluating nuclear factors which determine radiosensitivity." Thus as with energy absorbed per nucleus, when energy absorbed per chromosome (at lethal exposures) is considered, relatively small interspecific variation in radiosensitivity is found.

If this last conclusion holds true, it would then appear that the effect of polyploidy lies in increasing the number of chromosome arms rather than in increasing the redundancy of target information. Sparrow et al. (1965) showed that for the higher plants, no significant differences could be found in the energy absorbed per average chromosome at lethal exposure between the various members of a polyploid series (up to 22-ploid). This finding held for both woody and herbaceous species and suggested that in the multicellular meristem, gene deletion is unlikely to be a major factor in the production of radiation injury. Following this line of reasoning, one wonders at the meaning of the relationship between energy absorbed per chromosome and radiosensitivity; especially as the theoretical induction that led to the examination of this relationship was that an increase in the number of chromosome arms would result in a smaller proportion of the total chromosome volume being deleted. This implies that the crucial loss (with respect to reproductive integrity) is not genes per se, but rather a proportion of the total chromosomal volume (genetic information?) irrespective of the redundancy of information carried (unless all redundant information is inactivated - e.g. the "hot X" chromosomes in man - Gilbert et al., 1962; Rowley et al., 1963). This could make sense if all redundancies were inactivated and a damage sharing mechanism existed to spread

the energy among all the chromosomes, inactivated or not.

Van't Hof and Sparrow (1965) irradiated (single acute dose) dormant and actively growing Tradescantia roots and found that if they considered the energy absorbed per nucleus (dormant nuclei are larger) to produce an equivalent effect (reduction of growth rate to 37% of control), then the radiosensitivities were the same. From the observation that:

"Cytological and autoradiographic analysis of the actively growing and dormant roots showed that neither the number of cells in mitosis nor the number of DNA-synthesizing cells at the time of irradiation (my italics) could be correlated with the difference in radiosensitivity (in terms of radiation flux)" they conclude that,

"Because the proliferative capacities (my italics) of the dormant and actively growing roots at the time of irradiation were vastly different and because the amount of energy absorbed which produced an approximately equivalent effect in these roots was almost the same, an addition to the Bergonie-Tribondeau law seems necessary". However, it seems to me that although Bergonie and Tribendeau (1906) said that,

"les rayons X agissent (my italics) avec d'autant plus d'intensité sur les cellules que l'activité reproductrice de ces cellules est plus grande, que leur devenir karyokinétique est plus longue, que leur morphologie et leurs fonctions sont

moins définitivement fixées". The interpretation of this law has shifted the emphasis from the action of the X-rays to their effectiveness (Fletcher, 1959) or to the expression of their effects. In other words, their law was based on observations of the visible effects of irradiation, and is now taken to mean that faster growing (or dividing) tissues show the loss of reproductive integrity much sooner than do those tissues with a much slower turnover.

Van't Hof and Sparrow in focussing their attention on the reproductive activities (it is in this sense that they use the word capacities) of the two meristem populations at the time of irradiation, rather than on the period of growth following the irradiation, miss this point.

A better summary of these observations might be:

The susceptibility of a cell to the loss of its reproductive integrity is relatively constant when radiation dose is considered in terms of (units of) amounts of energy absorbed per nucleus and per chromosome. But because a sterilized cell will not be recognized as such until it is called upon to divide, and fails, the expression of this loss will be the sooner and the more obvious the greater the reproductive activity of that tissue during the post irradiation latent period. We have seen examples of this in the previous section on the problem of organismal radiation lethality.

It would appear then, that a knowledge of the mean DNA

content per chromosome would enable one to predict the dose necessary to produce severe growth inhibition. However, the experiments of Mortimer (1958) with the polyploid yeast Saccharomyces cerevisiae reveal a quite different relationship between ploidy and radiosensitivity (LD_{10}). Increases in ploidy above diploid, result in increased radiosensitivity. The endpoints used by Sparrow and co-workers (dose rate to yield severe growth inhibition) and Mortimer (LD_{10}) as measures of radiosensitivity are different, but this difference per se does not explain the apparent contradiction. Sparrow's data are consistent with chromosomal breakage and deletion as the cause of cell death; whereas Mortimer's data would seem to be explicable only on the basis of recessive and dominant lethal mutations. If Mortimer's interpretation is correct, a model relating induction of lethal mutations and cell survival curve parameters (my estimates from Mortimer's published curves - q.v. table I-1) would be of great value.

If we now look at those mammalian cells for which survival curves have been obtained, in vitro or in vivo, we find a range of chromosome numbers from 22 (hamster ovary) to 109 (L109 strain mouse cells), a five-fold increase. Under oxygenated conditions, ^{60}Co γ-ray and/or X-ray D_o's range from 117 to 290 rads, a three-fold increase, while extrapolation numbers vary between 1.28 and 20. Over this fairly wide range, q.v. table

I-2, the scatter is such that no correlation of the type suggested by Sparrow et al. can be found. Except for the results of Révész and Norman (1960), Silini and Hornsey (1962), and Bedford and Hall (1967), chromosome number seems to have little to do with radiosensitivity. In fact, considering the wide range of chromosome numbers and tissues investigated, the response of mammalian cells to X- and γ-irradiation has been remarkably uniform. Sparrow (1963) suggests that this might be due to the greater complexity of mammalian cells, but it is difficult to understand in what way a mammalian cell *in vitro* is more complex than a higher plant *in vivo*, in such a way as to produce the observed results. Moreover, is *Saccharomyces cerevisiae* more complex than for example *Chrysanthemum*?

Table I-1

Variations in Radiosensitivity with Ploidy in Saccharomyces cerevisiae

Ploidy	E*	D_o* (krad)	LD_{10} **
n	1	~2.6	~7
$2n$ (homozygous)	~2	~10.2	~30
$2n$ (heterozygous)	~3	~15.2	47.5
$3n$ 1	1.4 to 1.8	11.5 to 14.8	29
2			36
3			37.5
4			41
$4n$	~2	~10.2	30
$5n$	~1.4	~6.6	17.5
$6n$	~1.4	~6.6	16.8

* My calculations from the curves of Mortimer (1958).

** Mortimer (1958).

Table I-2

Cell Survival Curve Parameters for Mammalian Cells with Various Chromosome Numbers

Material	Mean Chromosome Number	Radiation Quality	$\bar{E}$	D_o	Reference**
HUMAN:					
Hela S-3	~78 aneuploid	230 kVp X-rays	2	138*	1
Conjunctival Liver Appendix) Epitheliod	~78 aneuploid	"	2	138*	2
Skin Spleen Ovary) Fibroblastic	~46 diploid	"	2	140*	2
HAMSTER (*Cricetulus griseus*):					
Ovary Clone A) ♀ Long V79-1) Fibroblastic	22 diploid	55 kV X-rays	4.5	128	3
			6.0-7.1	117	4
MOUSE (*Mus musculus*):	diploid=40				
Strain L, Cell line					
L_{53}	53	^{60}Co γ-rays	2	278 ± 16†	
L_{60}	60	"	2	290 ± 19†	
L_3	69	"	2	283 ± 17†	5
L_1	81	"	2	278 ± 28†	
L_{109}	109	"	2	270 ± 12†	

* Dosimetry corrected according to Morkovin and Feldman (1959).

† ± standard error.

** References

1. Puck and Marcus (1956).
2. Puck, Morkovin, Marcus, and Cieciura (1957).
3. Elkind (1960a).
4. " (1960b).
5. Till (1961).

B. The Choice of an Experimental Material

The behaviour of several anatomically important tissues has been explained, in the first approximation, by the use of stem cell models (e.g. Lajtha, Oliver and Gurney, 1962). Most such models have not been subject to disproof because of the lack of direct means for the estimation of stem cell population size. Furthermore, the influence of other, not totally independent, systems on the overall tissue behaviour has been shown to be such that indirect methods (e.g. LD_{50} determination) for the estimation of stem cell population size are of limited value.

In an experimental material lacking such complexities of interaction, and in which the stem cells can be counted, one should be able to determine the extent to which the simple model must be modified to predict the correct behaviour for even the simplest situations.

As we shall see in the second part of this introduction, the planarian is highly suitable for such studies. An additional advantage of the planarian is that natural euploid polyploidy allows one to study the effects of nuclear parameters on the radiosensitivity of an animal cell under *in vivo* conditions of normal growth regulation. As our present knowledge of the planarian stem cell (neoblast) - tissue interactions derives from the classical regeneration studies in this animal, we shall review the evidence upon

which the planarian stem cell theory is based before discussing the gross effects of irradiation on that animal.

The planarian is a choice material for the study of regeneration phenomena. Many authors have described the anatomical and histological processes of the regeneration of the principal organs of these animals. We know that the neoblasts (stem cells), dispersed throughout the parenchyma of the animal, are capable of differentiating to regenerate all of its organs; and that these cells migrate to the wound area. This migration is not spontaneous, but is initiated by a stimulus from the wound, which determines the direction of the migration (Wolff and Dubois, 1947 a and c). When the neoblasts reach the wound, they differentiate to replace the lost tissues (Dubois, 1949). This differentiation is known to be controlled by chemical factors which are produced by the differentiated tissues (Lender, 1956c, 1960). Normally, when regeneration is completed (both epimorphic and morphallactic stages) the planarian has recovered its anatomical and histological integrity.

C. The Planarian as an Experimental System.

1 Identification and Standardization

Planarians are triclad turbellarian platyhelminths and there are no uncertainties or disputes in the literature as to that part of their classification (order, class, phylum respectively). The suborder Paludicola contains the fresh water inhabiting triclads and this suborder is divided into three families: the Planariidae, Kenkiidae, and Dendrocoelidae, distinguished by their adhesive organs and the arrangement of the muscle fibres of the inner wall of the pharynx. The Kenkiidae are cave dwelling planarians and as they have not been of particular interest from the point of view of regeneration studies we shall not discuss them further. "The Planariidae lack adhesive organs, are pigmented or white, and have triangular to truncate heads". (Hyman, 1951) The Dendrocoelidae are mainly white planarians with two or more eyes (or none) and a truncate anterior end, often with a central adhesive organ. This family is abundantly represented in Europe by the genera Dendrocoelum and Bdellocephala and other genera, whereas in the Americas the only common dendrocoelid is Procotyla fluviatilis. Dendrocoelum and Bdellocephala are characterized in that the former possesses an adenodactyl whereas the latter lacks a penis papilla. At one time it was common to throw any two eyed planarid into

the genus Planaria, but this genus is now restricted to that species to which the name was first applied (Planaria torva O.F. Muller in 1776) and close relatives thereof. "As P. torva happens to have a peculiar type of adenodactyl (Kenk, 1930), very few planarians remain in the genus Planaria." The most commonly found genus to come from the former grouping Planaria is now called Dugesia (= Euplanaria), generally possessing two eyes, triangular or curved (rounded) head, projecting auricles and a dark pigmentation. Members of this genus are known for their strong powers of regeneration and frequently reproduce asexually by fission except during the seasonal sexual breeding period. Another genus, Polycelis is noted for its border of eyes along the lateral and anterior edges of the cephalic region. Polycelis is quite common in Europe, possessing several species, whereas only one species, coronata is to be found in the Americas (Hyman, 1951).

With the possible exception of Italy, the most common European species of Dugesia are the members of the lugubris-polychroa complex. They range from Sicily northwards to Denmark, Sweden, England, Scotland and N. Ireland (Benazzi, 1957; Maganini, 1961; Melander, 1963). Funaioli (1951) studying specimens from Italy, Germany and Sweden, using standard anatomical considerations, concluded that: "La presunta differenziazione tra Dugesia lugubris e Dugesia polychroa non possa essere accettata." Benazzi (1957, 1960a,

1965a) who is well known for his genetic and caryological studies of planarians, and his student, Pieragnoli (1953, 1954), showed that there exist at least 7 chromosomal bio-types of the Dugesia lugubris-polychroa complex, or more simply Dugesia lugubris in sensu lato of which four (A - D) form an auto- or allo-polyploid series.

Benazzi and Puccinelli (1961) published karyograms for the 7 biotypes. Biotypes A (as representative of B, C and D as well) E and G are all diploid 2n = 8 but the chromosome sets are not homologous, showing definite differences in length and morphology. F is diploid with 2n = 6, and its chromosomes are not homologous with those of biotypes A, E or G. Biotypes E, F and G are reproductively isolated among themselves and with respect to the A - D group, an observation which leads Benazzi and Puccinelli (1961) to suggest the existence of cryptic or incipient species inside of D. lugubris in sensu lato.

Table I-3 summarises what is known about the chromosomes of the autopolyploid series A - D, and the karyogram of the basic complement is reproduced in figure I-3.

Benazzi (1957) also gives the geographical distribution of the biotypes. Essentially, A has been found only in Italy; B all over Europe; C only in Italy; D very rare, even in Italy, but can be bred in the laboratory; E in Italy, France and Germany; and F only in Italy (and Sweden, Melander, 1963).

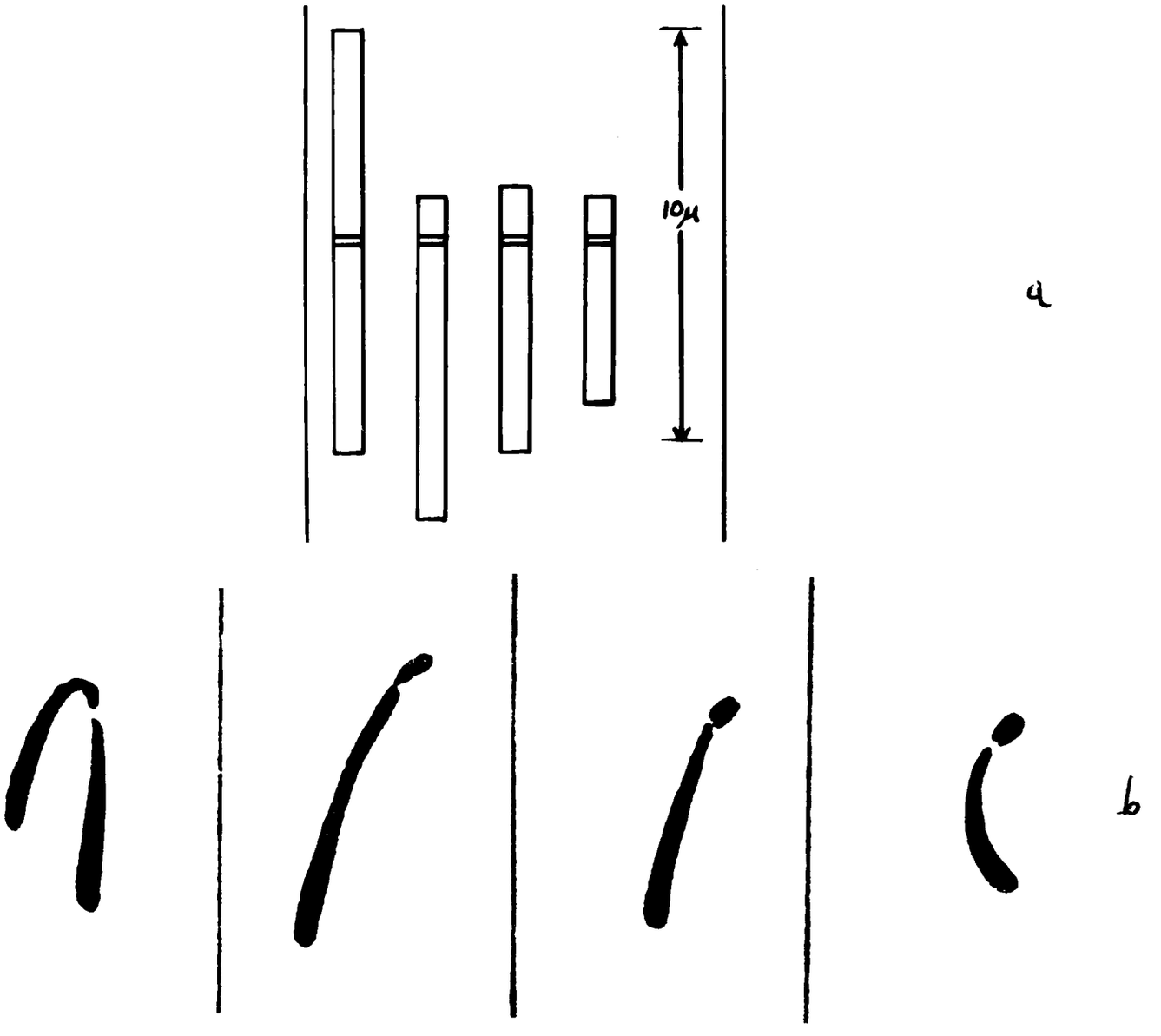

Figure I-3

Karyogram of the haploid complement of D. lugubris A, B, C and D

(a) Representation of size
(b) " " morphology /from Benazzi and Buccinelli (1961)/

The systematics of the Polycelis nigra-tenuis group is not quite as complicated. P. nigra (Ehr.) and P. tenuis (Ijima.) can be distinguished by the morphology of their penis papillae. Furthermore, P. nigra has no adenodactyl whereas P. tenuis occurs in two varieties: those with and those without adenodactyls. P. nigra is diploid with 2n = 16 (although a triplo-hexaploid line, like D. lugubris B, is also found in Italy) while P. tenuis is diploid with 2n = 14, in some areas, and 2n = 12 in others. Heteroploid lines of P. tenuis also exist. Hansen-Melander et al. (1954) suggest that the P. tenuis without adenodactyls should be made a new species, P. hepta, but Benazzi (1963a) disagrees as both P. tenuis and the proposed P. hepta, have the same basic chromosome set of n = 7. Furthermore, even P. nigra and P. tenuis which are morphologically and karyologically distinguishable, cannot be far removed from each other, as in laboratory conditions they are interfertile. Benazzi (1963) concludes that they are presumably two species in statu nascenti.

2 Regeneration

Regeneration in the planarian occurs in two stages which are comparable to those suggested by Needham (1952). The first stage consists of epimorphic regeneration which is biphasic and occurs in a relatively short period of time, and is

followed by morphallaxis which occurs over a much longer time period. The first, regressive, phase of epimorphosis consists of:

(1) wound closure,

(2) removal of damaged cells and defense against foreign noxa,

and (3) the provision of cells to provide new tissues for the progressive or repair phase.

This second progressive phase of epimorphosis is characterised by:

(1) the formation and growth of the blastema,

and (2) differentiation of the appropriate parts of the blastema.

The epimorphic stage is usually completed within a few days to weeks, while morphallaxis, the reproportioning of the animal, takes place over a period of weeks to months.

a Quantification

Child (1911b) and later Sivickis (1932), Watanabe (1935a), Abeloos (1930) and Brøndsted (1942a, b) performed the ground work of quantitative regeneration studies in planarians. The planarian head and its eyes are very distinctive parts of the animal. Thus a simple quantitative test for regeneration ability is to determine the percentage of animals which are capable of regenerating a new head (with eyes) after decapita-

tion. This proportion is known as the head-frequency. Child (1911b) introduced this method and later used it to test the effects on regeneration of various treatments.

Sivickis (1932) investigated the variation in head-frequency vs. position of cut on the long axis of the planarian body for several species of planarians. He and others wee able to demonstrate the existence of 5 different classes of regeneration ability (q.v. figure I-4).

However useful a tool head-frequency proves to be, one must not take head-frequency to be synonymous with regeneration ability.[2] We can also draw a curve of tail-frequency, for example, which shows that the postpharyngeal region of group 4 (Dendrocoelum) having 0 head-frequency, has 80 - 100% tail-frequency, i.e. has regeneration ability, As we shall see later, the difference in the shapes of these head-frequency curves is most probably due to differences in the competence of local tissues to be induced by the relevant hormones and thus to produce organs which were not formerly situated in that local tissue.

b What is the provenence of the cells which provide the new tissue (Anlagen)?

The ability of certain members of the Planariidae to regenerate lost parts of the body was known more than 150 years ago to several authors such as Pallas (1774)[1], Shaw (1791)[1],

1. Cited by Brøndsted (1955).
2. Brøndsted (1939).

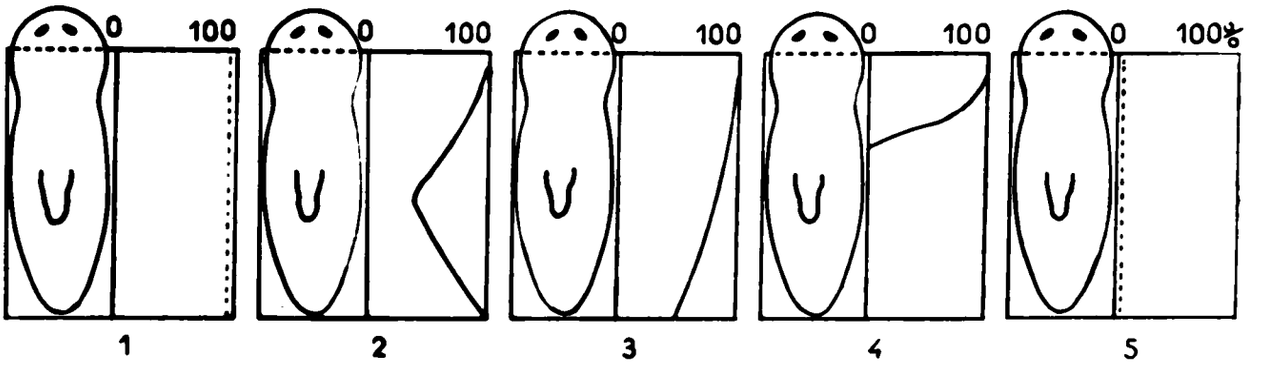

Figure I-4

Sivickis' five classes of head-frequency distribution (from Lender, 1965)

Group 1 Dugesia lugubris (100% regeneration)
" 2 Dugesia dorotocephala (progressive but not total loss caudally)
" 3 Planaria velata (maximum decrease in region of pharynx)
" 4 Dendrocoelum lacteum (or Bdellocephala) (no regeneration from pharynx region caudally)
" 5 Belloura (no regeneration).

Draparnauld (1800)[1], Johnson (1822, 1825)[1], Dugés (1828)[1], Faraday (1833)[1], Charles Darwin (1844)[1], Harvey (1857)[1], Zacharias (1885, 1886)[1], Wyman (1865)[1], and Schultz (1900)[1]. The early literature has been extensively reviewed by Randolph (1897) , Graff (1912-17)[1], Steinmann and Bresslau (1913) and Steinmann (1916)[1]; while the more recent literature (until 1955) has been excellently reviewed by Brøndsted (1955). I shall summarize the material reviewed by Brøndsted and then discuss the relevant current literature.

In the literature on Planarian regeneration a great debate has raged, for over 50 years, as to the origin of the cells which make up the regeneration blastema. Two fundamentally opposed views were put forward. The older, based on the Weissmannian concepts of purity of the germ line and tissue specificity, held that the parenchyma contains undifferentiated totipotent cells which retain their embryonic characteristics and serve as a reserve of totipotential cells which might participate in the seasonal development of reproductive organs, in asexual reproduction, or in regeneration, as need might arise. These cells have been called "Bildungszellen" (Wagner, 1890)[1], "Wanderzellen", Steinmann (1908), "Stoffträger" (carriers of nutrient material) with big nucleus and nucleolus (Lehnert, 1891) , "Stammzellen" (Keller, 1894) ,

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1. Cited by Brøndsted (1955).

"formative cells" (Curtis, 1902) or "néoblasts" (Randolph, 1897; Buchanan, 1933).

The term "néoblasts" was the name given by Randolph (1897) to describe the special mesodermal cells which play an important role in regeneration in the Oligochaetae. Buchanan (1933) adopted this term to describe the regeneration cells of the Planariidae without implying their source. The term "néoblasts" or "neoblasts" is now in common use amongst those studying planarian regeneration.

The other school held that the cells of the blastema arise from differentiated tissue cells by a process of dedifferentiation. This hypothesis was in keeping with Child's dynamic theory of the organism (Child, 1915 Introduction), which held that the essential feature of an organism (organic individual) is an axial physiological gradient. The differentiation of any part, being the expression of the conditions existing at that particular point along the gradient, is not irreversible. If the gradient is uhbalanced by the removal of a part, the controlling conditions are locally altered, resulting in local dedifferentiation. Child also refers to this gradient as morphogenetic, metabolic, or respiratory.

The evidence, from histological studies available at that time in favour of the stem cell hypothesis can be summarized as follows:

(1) Cells characterized by a compact cytoplasm and a darkly staining nucleus with a large nucleolus are involved in regeneration in many of the invertebrates that possess extensive powers of regeneration (Abeloos, 1932, Stolte, 1936) and are normally present even when the animal is not in the process of regeneration.

(2) These cells are associated with asexual reproduction by budding and fission, and with seasonal development of sexual organs (Curtis, 1902).

(3) In the planarians, those species possessing them most abundantly regenerate most readily (Curtis and Schultze, 1934) and are usually those which reproduce by fission. The objection (Hyman, 1951) that there is no marked decline in the number of neoblasts in those regions of Procotyla fluviatilis which can not regenerate a head, but can regenerate a tail and copulatory apparatus, can be countered with the hypothesis that it is possible that the ability to form new tissue is dependent on the number of neoblasts; but the ability of this new tissue to form the correct organs depends on the surrounding differentiated tissues.

(4) In planarians, the neoblasts are the only cells known to undergo mitosis, and their mitotic activity and numbers are greatly increased during regeneration (Flexner, 1898).

(5) They begin to appear at the site of the injury with-

in a very short time (< 12 - 24 h). (Flexner, 1898)

(6) The elongated shape and orientation in the direction of the wound have been interpreted as evidence of migration to the blastema.

No data incompatible with the blastema arising from stem cells have been put forward by Child's school, their objections being based on the alleged incompatability of the stem cell concept with Child's gradient hypothesis. Moreover, although the concept of a gradient, of head-frequency or of regeneration ability, is most useful for the explanation of the myriad results of the multitude of regeneration and grafting experiments performed, the gradients do not correspond to any metabolic gradient in terms of respiration rates (Petrucci, 1962), a correlation which Child insisted upon. Brøndsted has developed Child's concept of an axial gradient by admitting that the morphogenetic or head-frequency gradient is not known to be correlated with any other type of gradient that might exist in the animal, and by further extension of this axial gradient concept into that of a "time-graded regeneration field."

As late as 1941, Wilson wrote, "It is clear that evidence which seems conclusive to one group fails to carry conviction to the other. Until some essentially new approach is discovered we can only examine and weigh the evidence and withhold

judgment."

In 1946 Verhoef, studying the mitotic activity during the regeneration of Polycelis nigra, found mitoses in the parenchyma both of normal and regenerating animals. She was unable to find any diurnal variations in mitotic index, and found, for the first 48 hours (1 - 2 hour intervals), the mitoses evenly distributed through the (parenchyma of the) whole body. After 48 hours she observed an increasing concentration of mitoses at the base of the regeneration bud. By taking the percentage of mitoses per half animal in each of four equal (length) transverse divisions (each half animal → four divisions; whole animal → eight divisions), she was able to show an increase in the percentage of mitoses in the division proximal to the wound (and corresponding decreases in those distal to the wound) starting 48 hours after the cut and continuing to increase for two days thereafter.

A new approach was made by Wolff and Dubois (1947 a, b, c, 1948 a, b) and carried on by Dubois (1948 a, b, c, 1949, 1950, 1951)[1] with the result that now there is no doubt that the neoblasts are the only cells to contribute significantly to the cellularity of the planarian regeneration blastema. The only authors who still insist on dedifferentiation (Chandebois; Burnet and Woodruff) have not yet presented any data incompatible with the stem cell hypothesis (q.v. follow-

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1. After 1950, Stéphan-Dubois.

ing sections).

Dubois' new approach was to use localized irradiations followed by wounding, or total-body irradiations followed by grafts of unirradiated tissue. By these means she was able to demonstrate the following effects after X-ray (50kV) doses sufficient to prevent regeneration (>3.5kr, usually 5 - 8 kr):

(1) The short-term histological picture remains normal except for the cytolysis and disappearance from that region of nearly all the neoblasts. This was a qualitative observation, confirming those of Weigand (1930) and Curtis and Schultze (1934).

(2) Successful regeneration after a cut through an irradiated region takes place only after neoblasts have migrated from the shielded portion of the animal, or from the graft, to the wound surface.

(3) Repair of post-irradiation necrosis takes place as, and only when, the neoblasts pass through the irradiated region.

(4) The pattern of mitotic activity in the irradiated regions of the planarian follows that of neoblast appearance in these regions.

(5) Neoblast migration is initiated by a cut or puncture through the epithelial layer of the planarian, and migration takes place only in the direction of the wound. However, note the findings of Fedecka-Bruner (1964) mentioned below.

(6) If the irradiated host and unirradiated donor are of different pigmentation, the regenerate takes on the colour of the graft (Benazzi, 1961, 1962a has shown pigmentation to be a genetic trait controlled by at least three alleles) further indicating the source of the cells repopulating the neoblast-depleted host.

(7) The differentiation control determining what organs are to be regenerated, and their polarity, is determined by the host tissue (provided that the graft does not contain a brain) even after doses of 8kr.

Dubois found these results to hold for D. lugubris, D. tigrina, and Polycelis nigra.

c What controls the differentiation of the neoblasts - are they predetermined or is the control exerted locally, or at a distance, by the differentiated tissues?

In this section we shall consider evidence relevant to the support or refutation of the hypothesis that neoblasts are (1) totipotential and (2) differentiate under the control of hormones(s) produced by differentiated tissues.

Stéphan-Dubois and Kolmayer (1959), using the method of partial irradiation, showed that in D. lacteum, irradiated prepharyngeally, (case A, the anterior half of that region; case B, all of that region) and then decapitated, the irradiated neoblasts form an abortive blastema which rapidly regresses,

Subsequently radiation lesions appear and finally disappear and recolonization from postpharyngeal neoblasts occurs. After recolonization, the blastema is reformed and a normal head is regenerated from the postpharyngeal neoblasts. In 1960 Stéphan-Dubois and Gilgenkrantz, by means of grafts, reconfirmed that in D. lacteum the neoblasts of the tail (postpharyngeal) region can give rise to either a head or a tail (i.e. are totipotential[1]), even though the postpharyngeal region cannot itself regenerate a head, and that thus the neoblast potentialities are seen to be determined by the tissues in which the blastema develops: "chaque territoire conserve donc ses potentialités propres." Kolmayer and Stéphan-Dubois (1960) showed that the head-frequency curve of D. lacteum is determined by the local differentiated tissues and not by the neoblasts. However, when the neoblasts had to migrate 40% of the body length to reach a point of 100% head-frequency (for 0% or 20% body length migration) head-frequency was only 80% Similarly, for a point of 70% head-frequency for no migration, a head-frequency of only 50% was obtained after 20% body length migration. These differences may not be significant, but even should they prove to be so, the decrease in head-frequency could be due to differentiated

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1. Another possibility is that each individual neoblast is predetermined and neoblasts of all types are randomly distributed throughout the animal. However, in view of the local differentiation control exerted by the differentiated tissues such a dual system of control is unnecessarily complex to explain the extant data.

tissue deterioration during the longer time needed by the healthy neoblasts to traverse the greater distance.

Fedecka-Bruner (1964) has shown, by using low energy X-rays, that regeneration of the testis of D. lugubris is accomplished by the neoblasts and that an internal lesion (in contradistinction to Dubois' finding that only an external lesion) is sufficient to cause migration (over relatively short distances, 5-600μ) to the site of the damage, there to undergo mitosis and differentiation. Thus the spermatogonia which eventually become spermatids and spermatocytes are shown to be derived directly from the neoblasts. As the neoblasts do not start to mass until about 10 days post irradiation for this regeneration, it may be that in the case of Dubois' partial irradiation, the 10 day delay (or more) in the onset of migration to a site of internal damage plus the long distances that had to be traversed, meant that the neoblasts did not arrive in the irradiated parts until too late. Also it might be postulated that the site of internal damage can only set up a weak (localized) field of migration call, whereas an external wound site can build a far-reaching field of call.

The study of pharynx regeneration was the first example of the inductive mechanism in planaria. Santos (1929 and 1931) showed with D. dorotocephala and D. macculata that grafts of a head into the postpharyngeal region produce the

differentiation of one or two supernumerary pharynges in the host tissue which are of the same polarity as the graft. Santos suggested that the inductive organ might be the brain. Chevtchenko (1936) confirmed Santos' findings in D. lugubris. Sugino (1940 and 1941) studied the induction of pharynges in different levels of the body in D. gonocephala by grafting heads into the prepharyngeal, pharyngeal, or postpharyngeal regions. The prepharyngeal region did not develop a pharynx, but the other regions did. Okada and Sugino (1934 and 1937) and Okada and Kido (1943) showed that the power of pharynx induction was not limited to the cephalic region. In D. gonocephala the prepharyngeal region, grafted posteriorly caused the development of a supernumerary pharynx. Sengel (1951a) showed that D. lugubris, with its head, prepharyngeal region and its pharynx cut away, could still regenerate a pharynx within the pharyngeal region. Thus the pharyngeal region itself could regenerate a pharynx within its own tissues. Sengel (1951b) repeated Santos' experiments in D. lugubris, with the same results. The grafted head caused a pharynx to differentiate in the tail region. Removing the graft and the supernumerary pharynx, Sengel found that the supernumerary pharynx is regenerated in the complete absence of any graft tissue. Thus Sengel (1953) concluded that the regeneration of a pharynx is a double induction. The grafted

anterior regions induce the irreversible formation in the host tissues of a pharyngeal zone, which is then capable of inducing the regeneration of a pharynx. This would seem to support Brøndsted's concept of a morphogenetic gradient, the more "active" region inducing the less active one.

The second example of induction in planaria was the study of eye regeneration.

Wolff and Lender (1950) showed that when a lateral block of eyes is cut out, the first part of the eye to regenerate, the pigmented eyecup, appears on the 7th day in Polycelis nigra. If at the same time, one removes

(1) an anterior block of eyes,

or (2) the brain,

regeneration is not retarded. Histologic preparations show that the regenerating brain is clearly identifiable on the 3rd day. However, if after ablation of the brain and a lateral block of eyes, the regenerating brain is repeatedly excised every 2-3 days, then the eyes do not develop until 7 days after the last excision. Thus there would seem to be some connection between the brain and eye formation. Lender (1950) using a grafting method, concluded that in P. nigra the brain is capable of inducing the regeneration of pigment cups in a receptive area (one which is normally occulated), but that this organizing power is felt only in regions near the brain.

In a major paper, Lender (1952) showed that:

(1) The eyes of D. lugubris and P. nigra are similar, but those of P. nigra are simpler. During regeneration of the eye, the eyecup appears first, followed by the differentiation of visual cells, with the regeneration of the optic nerve coming last.

(2) At 18°C, the excised eyes of P. nigra regenerate on the 7th day; the delay being independent of the mode of operation.

(3) The excised eyes do not regenerate in the absence of a brain. Localized irradiations of the brain destroy the neoblasts in that region, nevertheless eyes regenerate on the posterior lateral border (shielded area) after the usual 7 day delay, and on the anterior border after a longer delay (~13 days) by which time intact neoblasts have migrated to that region. Thus the induction acts on neoblasts at a distance.

(4) Homologous grafts demonstrate that the inductive action of the brain is not transmitted by the nerves and that the field of organization of the brain extends along the edges of the animal, as far as the level of the posterior most eyes, and along the middorsal line to the level of the mouth. The brain grafted into the posterior region of the body gives rise to an inductive field such that the oculated border within it (the graft) regenerates its eyes.

(5) Grafts of the non-ocḱulated border demonstrate the existance of limits to the region receptive to the inductive action of the brain. This region is limited to the normally ocḱulated edge.

(6) The brain need not be physiologically or histologically intact to exert its inductive power. A degenerating brain (1 month after >3kr X-irradiation) still acts as an inducer in the regeneration of eyes.

(7) Heterologous grafts show that the inductive power of the brain is not species-specific. The brain of D. lugubris induces differentiation of eyes in a graft of the ocḱulated edge of P. nigra.

From this Lender concludes that the brain induces eye formation in P. nigra during regeneration, acting via chemical 'organisines'[1] which diffuse throughout the body of the planarian; and that these hormones induce the regeneration of eyes in the receptive areas by acting on the neoblasts.

By 1956 Lender (1956a) was able to show that:

(1) A mince of planarian heads added to the regeneration media allows the regeneration of eyes in the absence of a brain, whereas a mince of tails does not.

(2) A cell-free extract of heads or tails allows the regeneration of eyes in the absence of a brain.

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1. This is Lender's term, however the existing terms "hormone" or "humoral factor" express the same meaning in English.

(3) The active substance (the hormone(s)) of the extracts is not species-specific and is not inactivated by 70% or 95% alcohol, nor by heating to 60°C for 2 minutes. However, stronger heating reduces the activity of the hormone(s).

(4) The hormone(s) is present in the posterior regions of the animal but cells must be destroyed to release it. Extracts from these regions are much weaker (less concentrated) than those from the head.

(5) It is sufficient for the hormone(s) to act on alternate days or for only two consecutive days (optimally the 3rd and 4th days after the operation) during regeneration, to induce eye formation in the absence of a brain.

Thus Lender concluded that the regeneration of eyes in P. nigra is induced by a hormone(s), a chemical substance produced by the brain and present throughout the body of the planarian but in a concentration gradient decreasing caudally. And furthermore, that in the posterior region the hormone(s) is immobilized within the cells.

Lender (1956b and 1960) also points out that these cell-free extracts specifically inhibit the formation of a brain in the regenerates and that the inhibition is not species-specific.

Thus we have three examples of how differentiation is controlled in the planarian (Lender, 1956c).

(1) The induction of a zone which is then capable of inducing specific organs as seen in the case of pharynx

regeneration.

(2) The induction of a specific organ (the eyes) by chemical means, i.e. by a hormone(s).

and (3) The inhibition of the formation of a specific organ (the brain) by chemical means, again by a hormone(s).

Therefore planarian regeneration has been shown to depend in part upon hormones.

Examples 2 and 3 were further investigated by Lender and in 1961 two new pieces of information were added. Secretory cells, stainable with Gabe's paraldehyde fuchsin, were identified mainly around the periphery of the ventral surface of the brain. Their number was found to increase almost three fold by the 3rd and 4th days of regeneration and then drop to about half the control value by the 6th day. (Lender and Klein, 1961). Further refinement of the hormone(s) showed that it remained in the supernatant after centrifugation at 10,000 rpm (20 min) but sedimented after centrifugation at 20,000 rpm (20 min). The same effect was found to be produced by a substance extracted in the same manner from 9 day chick embryo extract (Lender and Deutsch, 1961).

We shall have cause to return to the question of the nature of the hormone(s) at the end of the section on planarian metabolism.

Fedecka-Bruner (1961) may have demonstrated yet another differentiation control factor in the sexually reproducing

planarians. By a series of excisions and cuts or local irradiations, she showed that the copulatory apparatus of D. lugubris is formed from neoblasts and that the delay in regeneration is longer, the more that tissue is cut away. Thus it is confirmed that, what was known from previous attempts to induce sexuality by temperature changes, (Hyman, 1941, 1943) in order for a copulatory apparatus to differentiate the animal must possess a certain threshold amount of tissue.

Le Moigne (1965a) has shown that the embryo of Polycelis nigra is capable of regeneration at stage V or later in its development. As Le Moigne points out, the posterior pieces of stage V and VI embryos have no nervous tissue. Thus it might appear that Singer's hypothesis (Singer, 1952) that regeneration is mediated by the nerve tissue (in the Vertebrata) does not hold for planaria. However, further studies by Le Moigne (1965b, 1966), and Teshirogi and Jin (1964), show that nerve tissue differentiates before the blastema can differentiate.

The one finding, apparently contradictory to the above interpretation of the extant data, was that of Brøndsted (1939). He had shown, in D. dorotacephala and D. gonocephala, that the polarity of a host remained unaffected by the graft of a head (fully differentiated or still regenerating) in reversed polarity, i.e. dorsoventrally or antero-posteriorly. The graft head was found to exert an organizing influence on

the host only if

(1) the head was still in the process of regeneration, and (2) was of the same polarity as the host. Furthermore if a freshly cut anterior piece (possessing head-forming ability) was grafted in reversed polarity to a freshly cut posterior piece (possessing tail- not head-forming ability) and then cut away 2, 3, or 4 days later, the tail piece still could not regenerate a head, i.e. no head-organiser substance ("Organisatorstoffe") passed from anterior piece to posterior piece. After further experiments, Brøndsted (1942a) concluded that "during regeneration organizing power is transmitted from cell to cell and is not due to freely circulating 'Organisatorstoffe'."

However, the apparent contradiction disappears if we allow that firstly, polarity could not be reversed in Brøndsted's experiments because the prepharyngeal postcephalic region was already induced with a polarity. Secondly, the hormone(s) may not diffuse freely but may have a preferred direction of transit /as suggested by the data of Marsh and Beams (1952) on electrophoretic reversal of polarity in regenerating pieces of planarians/. Thus, although introduction of hormone(s) into the milieu was most effective for a two (or longer) day exposure including days 3 and 4 post decapitation, diffusion from the milieu, across the wound surface, into the decapitate may have been greater than from the reversed-

polarity graft into the host.

Thus the extant data are compatible with the hypothesis that neoblasts are totipotential and differentiate under the control of hormone(s) produced (and transported?) by differentiated tissues.

d What is the origin of the neoblast? Is it an embryonic cell or does it result from dedifferentiation of somatic tissues?

Le Moigne (1963) studying the embryonic development of Polycelis nigra from cocoon deposition (prior to first cleavage, Lepori, 1949a and b) to hatching (at 18°C), observed, in serial-sectioned material, that the blastomeres multiply actively, that they give rise to all of the differentiated tissues, and that they disappear on the appearance of the neoblasts of the adult during what he calls stage V (day 9-14) of embryonic development. He gives a detailed description of the order of appearance of the various organs and the state of their development at each of the seven stages into which he divides embryogenesis. With the exception of one point, in the development of the pharynx, he also found the same processes of development for diploid and triploid Polycelis tenuis. As a result of these observations, Le Moigne concludes that the concept of triploblastic development does not apply to planaria as all tissues derive from one cell line, the blastomere, or later the neoblast.

A comparison of ultrastructure (Le Moigne, Sauzin and Lender, 1966) shows great similarities and some small differences in detail between the neoblast and the embryonic cell, the blastomere.

In the adult D. tigrina, Woodruff and Burnet (1965) suggest that the neoblast derives by dedifferentiation from the gastrodermal gland cells. The observations upon which they base this conclusion are threefold:

(1) In regenerating and control animals the intestine shows isolated areas of destruction, the greatest degree of which occurs near the wound region. This degeneration increases until day 4, the time at which the maximum number of neoblasts are to be found.

(2) This degeneration is accompanied by RNA-rich intestinal gland cells showing morphological changes and disappearing from the intestinal wall.

(3) They presume that mitosis in neoblasts cannot account for the large numbers of these cells in regenerating worms (no quantitative data are given, just the statement that mitotic figures are rare).

Similarly, Chandebois (1962, 1963b, 1965a) reports a general degeneration of the parenchymal 'syncitium' (although Pedersen's (1961a) electron microscopic studies of P. vitta and D. tigrina show the cellular nature of the parenchyma) but makes no mention of intestinal degeneration in Dugesia

subtentaculata.

On the other hand, Lender (1960), and Gabriel 1961 Stéphan-Dubois (1949), Pedersen (1958, 1959a, b, 1961a, b), Skaer (1961, 1965), Teshirogi (1962)[2] and others make no mention of either type of degeneration and some (Lender and Stéphan-Dubois)[3] categorically state that no degeneration occurs during regeneration in their material. It is conceivable that these discrepancies are merely species variations, but in view of the great similarities within the Planariidae and even extending to the fresh-water triclads in general, this seems unlikely. Chandebois' histological observations are almost completely based on alcohol-fixed squashes[1], a technique which Pedersen (1961a) points out is not without risks. The fixative of Woodruff and Burnett (Helley) is quite unimpeachable for their serial-sectioned material. However, they do not state for how long the animals were fasted prior to fixation. Hyman (1924) has shown that food in the planarian gut can cause, on fixation, quite serious damage to other tissues. On the other hand, if the gut is genuinely damaged or degenerating (even in the normal animal?) then it would not be surprising to find neoblasts on the intestinal border in the process of repairing it. As far as the statement that neoblast mitosis cannot account for the observed increase,

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1. Chandebois also used OsO4 fixed material for electron microscopy but this fixative can also produce artifacts - q.v. last paragraphs of this chapter.
2. Cit. Stéphan-Dubois (1965).
3. In the discussions in Regeneration in Animals and Related Problems, ed. V. Kortsis and H. A. L. Tramrusch, North-Holland Publishing Co., Amsterdam (1965).

such a conclusion cannot be reached without quantitative data.

Melander (1963) in a cytogenetic study of embryogenesis in Polycelis tenuis by means of squashes, observed that over the first 82 hours (at 23°C) the proportion of intact free blastomeres in mitosis did not change significantly. He suggests a mitotic cycle time (cell cycle time) on the basis of the proportions of cells observed in the various phases of the mitotic cycle and the rate of increase of free blastomeres, of approximately 8 hours 47 minutes, of which mitosis (prophase through telophase) occupies 37 minutes or about 7% of the cycle. After the first 82 hours (~10 cell doublings) a rapidly increasing number of blastomeres undergo morphological changes which may be indicative of differentiation. The degenerating cells observed by Melander, may well be the degenerating vitelline cells described by Le Moigne (1963).

Assuming that the neoblasts have the same cycle time as the blastomeres, and noting that Lender and Gabriel (1961) observe 0.5% of the neoblasts in the body and 5% of those in the blastema of the regenerating planarian in mitosis, then we see (table VI-4) that with almost three cycles per day more than enough neoblasts could be produced by mitosis alone, to account for the increased number of neoblasts observed (Lender and Gabriel, 1960) at various times after cutting.

Thus the observations of Woodruff and Burnett and of Chandebois are not, in fact, incompatible with all neoblasts deriving directly from embryonic cells (blastomeres) by mitosis.

In figure I-5 we have a résumé of the relationships between the neoblasts and the differentiated tissues of the planarian.

3 Metabolism and Polarity

a Digestion and Excretion

Jennings (1957) in a detailed study of feeding and digestion in Polycelis cornuta (and several other Turbellarians) found that food is broken up into small particles during ingestion by the muscular activity of the pharynx (no enzymatic breakdown). No evidence for any (gut) intraluminar digestion of carbohydrates, proteins, or fats was found. Food particles of up to ~30 μ were phagocytosed by the columnar gut cells, and digestion (of carbohydrates, proteins and fats) proceeded intracellularly. A certain amount of the phagocytosed material was passed back into mesenchymal (parenchymal fixed cells) cells for digestion. Large reserves of protein (in the "sphere" cells) and fat (globules in inner mesenchyme and columnar gut cells) and small reserves of carbohydrate (glycogen granules throughout the mesenchyme and columnar cells) were found in the well fed Polycelis. Upon starvation, the glycogen disappears rapidly

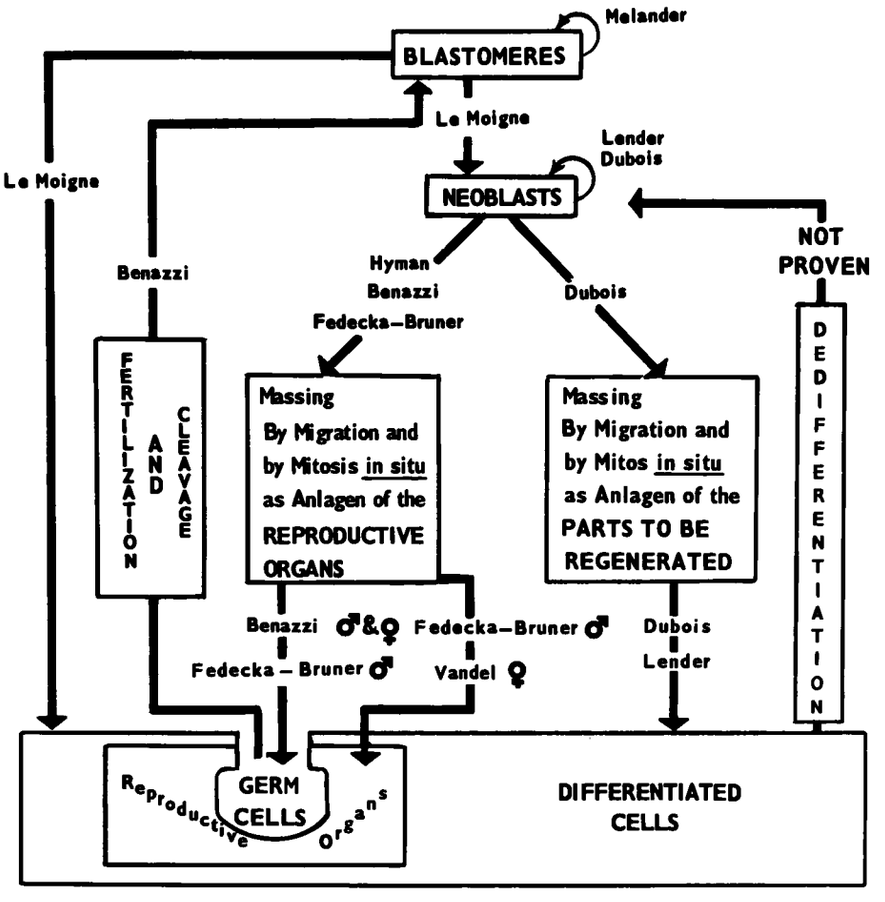

Figure I-5

The evidence for the stem cell nature of the neoblast

The names on the arrows refer to the authors (or schools) who demonstrated the relationship represented by that arrow.

and is absent by the end of a fortnight (q.v. Hyman's respiratory changes in starvation); the ratio of "sphere" (protein storage) to columnar cells decreases (from a plot of his data, linearly with time); whereas the stored fat decreases relatively much more slowly. Rosenbaum and Rolon (1960) identified the digestive enzymes of D. dorotocephala and tigrina, as acid phosphatase, aminopeptidase and β-glucuronidase. They also confirmed Jenning's observation of some phagocytosed material being passed back to mesenchymal cells for further digestion. Aminopeptidase activity was found to increase in the mesenchymal cells with increased starvation, and was (during starvation) totally absent from gastrodermal cells. This they suggest is the means for the well known "self-digestion" and shrinkage of starved planarians. Thus, in extremis, the planarian can utilize its own body tissues as food reserves.

Indigestible food particles are returned to the gut lumen by the phagocytic gastrodermal cells and the gut contents are discharged by the intake of water (through the pharynx) and then the flushing out of the gut contents by violent contractions of the body.

Metabolic wastes (Pedersen, 1961a, b) are transferred to the protonephridia of the flame cells, and excreted through pores in the body wall.

Pedersen (1961a) suggests, on morphological grounds,

that the fixed parenchyma cells[1] may have a function as a "mediator of transfer of metabolites", as well as their role as a supporting tissue.

b Respiration

As has been mentioned above, Child's theory of a metabolic and, in particular, a respiratory gradient to explain the observed gradient in head-frequency, was not based on rigorous measurements of respiratory and metabolic rates. Child (1948) did observe a gradient of uptake of various vital stains (e.g. Janus green, Methylene blue, Neutral red, Brilliant cresyl blue, Nile blue sulphate, and the indole reaction) which he interpreted as being indicative of a respiratory gradient.

Allen (1919a) using a modification of Winkler's method, found that up to 80% of the normal oxygen consumption of D. maculata (D. tigrina) and P. agilis can be reversibly inhibited by KCN. The other 20% of the normal oxygen consumption is unaffected by increases in KCN concentration. Hyman (1919a) using the same method presents detailed quantitative data on the relationship between KCN concentration and oxygen consumption in D. dorotocephala.

Hyman (1919b) found that after the ingestion of food, D. dorotocephala and P. velata exhibit a marked rise in oxygen

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1. "There is no doubt about the fact that these two cell types /fixed parenchyma cells and neoblasts/ are completely different. This is borne out by morphological, histochemical and functional considerations."

consumption (Winkler's method). This increase lasts for several hours (7-8) then starts to fall, and by 24 hours has dropped markedly. The decrease continues gradually until it is back to normal by 2-7 days and, with continued starvation, continues to fall until day 14. Thereafter, oxygen consumption rises again and after prolonged starvation (50 days) is much higher (~ 2-5x) than in animals starved only a few days. If specimens of D. dorotocephala are cut into small pieces, the oxygen consumption per unit weight increases (Hyman, 1919c). This increase persists through about 48 hours after which respiration drops and remains low for about a week (due to starvation), but the decrease is less than that for non-regenerating starved worms. Thereafter, oxygen consumption rises and continues to rise as regeneration proceeds until the worms are completely regenerated and have an oxygen consumption rate of 50-150% greater than that of the original worms. Upon refeeding, the oxygen consumption rises still higher.

D. dorotocephala young produced by fission consume up to 55% more oxygen per unit weight than do their adults. P. velata young produced by an asexual process (fragmentation, encapsulation and regeneration) consume twice as much oxygen per unit weight as do their adults, and sexually produced young of P. maculata (D. tigrina) consume 50% more oxygen per

unit weight than do sexually mature worms. Thus young planarians, irrespective of the mode of their production, have a higher metabolic rate than do adults (Hyman, 1919).

The metabolic rate of the planarian is known to increase with youth, regeneration and feeding as well as with increased temperature and movement and to fall with low temperature, acidity and during the early phases of starvation (Hyman, 1920). The increase in metabolism associated with prolonged starvation is due to the fact that such animals are no longer living on their food reserves, but are feeding again, using their own tissues for food. Hyman (1920) has also shown that the pattern of starvation is a function of species as well as of milieu, e.g. "P. agilis has a lower metabolic rate, greater food reserves, and loses body weight more slowly in starvation than other species. For this reason it must be starved for a longer period before its rate of oxygen consumption increases" due to its consumption of its own tissues.

Hyman (1923), using the method of Winkler, found small but apparently consistant differences in the respiration rates of pre-, post-, and pharyngeal regions of D. dorotocephala. However, Løvtrup (1953) using the 'cartesian diver' technique, was unable to show evidence of a respiratory gradient in D. lugubris. The latter's results are not inconsistant with those of Hyman, but Løvtrup shows the observed differences

to be not significant, yielding a mean respiration rate of 10 $\mu l O_2 hr^{-1} mgN^{-1}$. Marsh (1962) also found no significant difference between O_2 consumption at various levels. Moreover, he states that analysis of Hyman's data (1923) also reveals no significant differences. Petrucci (1962) in a detailed and rigorous study of respiration and respiratory enzymes in three species of planaria having three different head-frequency gradients (D. lugubris, P. torva, and Dendrocoelum lacteum), was not able to find significant differences in cytochrome oxidase or succinic oxidase activities in prepharyngeal, pharyngeal and postpharyngeal pieces of any of the three species. He did however demonstrate the presence in planarians of the respiratory enzymes mentioned above, as well as the capability of the mitochondrial fraction to carry on the tricarboxylic acid cycle (in vitro), and that this cycle is carried on in vivo.

Løvtrup (1953) found, contrary to Hyman's observations for D. dorotocephala and P. velata, no change in respiration over the first 6 days of head regeneration in D. lugubris. Pedersen (1956), using the cartesian diver, has also shown that oxygen uptake per total nitrogen does not change during the first 24 hours after cutting or at fission of P. vitta. Moreover, the respiration of 30 and 65 day starved animals is not different from that of nonstarved controls (8.4 $\mu l O_2 hr^{-1} mgN^{-1}$). As total N for P. vitta is reduced to 80% after 30 days and to

60% after 60 days starvation, there can be no question but that the animals are shrinking. Pedersen compares animals of approximately equal size (mg. N) at the time of the measurement, whereas Hyman was comparing animals of same size at start of the experiment. This may account for the differences in the reported respiration rates. Hoff-Jørgensen et al. (1953) found that 60 days starvation reduces Polycelis nigra to 14% of its original total N, but no significant ($0.10 > P > 0.05$) decrease in DNA was observed.

o Other Metabolism

Coward, Flickinger and Garen (1964) found an increased rate of regeneration ($^{14}CO_2$ uptake during eye formation) in the posterior postpharyngeal region (not elsewhere) of starved D. dorotocephala exposed to a nutrient medium containing, amongst other things, 1% chick embryo extract. They also present some data which suggest a stimulation of regeneration rate (eyes in 14 days starved animals) by 5×10^{-5} w/v yeast sodium ribonucleate. This is consistent with the report of Brøndsted and Brøndsted (1953).

Coward and Flickinger (1965) also studied the relative distributions of protein and RNA synthesis at 8 levels along the cephalocaudal axis of D. dorotocephala. In the intact worm, protein and RNA synthesis are maximal in the head. RNA synthetic activity drops to 60% of the head value just behind the auricles and remains constant caudally. Protein synthesis,

however, drops to 40% of the head value in the postcephalic and pharyngeal regions, and then rises sharply to 60-70% of the head value in the postpharyngeal regions. It is difficult to evaluate their claims regarding variation in ^{14}C - algal protein hydrolysate or leucine-1-^{14}C uptake during regeneration as they do not give standard errors. However, for ^{14}C-uracil uptake they present their data. Summing over pieces 1-6, we find no significant difference in ^{14}C-uracil uptake in the regenerating or in the intact animals. Unfortunately, variations between determinations at each level are such that, with the number of determinations made, only very large changes in distribution are found to be significant.

Best, Rosenvold, Souders and Wade (1965) studied the uptake of ^{3}H-thymidine and ^{14}C-L-leucine in D. dorotocephala and found almost no uptake unless the labelled compound was given in the form of digestible particles (e.g. baker's yeast). They determined the amino acid composition of the proteins of D. dorotocephala, of which histidine, argenine and lysine are in greatest quantity, and estimated a minimum protein turnover of 46% in 7 weeks. Pedersen (1958) using cytochemical methods demonstrated the presence of argenine, lysine, and possibly histidine in the néoblasts. Protein turnover ~~times~~ estimates from incorporation of ^{14}C-glycine disolved in the medium (Flickinger, 1963) are less than 10% of those of Best et al., 1965. The latter authors suggest that this is due to the low rate of exchange of amino acids between the solution and the

planarian's metabolic pool. Thus the axial gradient in ^{14}C-glycine observed by Flickinger (1963) may reflect differences in permeability rather than rate of protein synthesis. Furthermore, they suggest that the high concentrations of ^{3}H-thymidine employed by Flickinger (1964) to demonstrate that neoblasts do not migrate (from a graft into the host's irradiated tissues) were sufficient to sterilize the graft's neoblasts. Lender and Gabriel (1965) show the migration of ^{3}H-uridine labeled neoblasts from a graft to the regeneration blastema. They also find, 2-3 weeks after decapitation, that some of the differentiated cells in the regenerate are labelled but have a lower grain count than do the neoblasts.

Chandebois (1963a) in a quantitative study of the amino acid composition of Dugesia subtentaculata (gonocephala group) by means of paper chromatography, found a complete absence of arginine (which is toxic to fragments of D. subtentaculata in culture) and extremely low concentrations of histidine and lysine, the three most common amino acids of D. dorotocephala. Serine was the amino acid in greatest quantity for D. subtentaculata.

Kohl and Flickinger (1966) report that D. dorotocephala, exposed to 1 mg/ml of chloramphenicol for 7 20 hours before cutting, produce bipolar (heads) animals (2/20 bipolar in 20 hr exposure group; 8/20 in 24 hours exposure group). A 24

hour exposure to the same concentration of chloramphenicol, they say (again no standard errors), also produces a 89% inhibition of DNA synthesis (^{14}C-thymidine uptake). Incubation of head-and tail-forming blastemata and the regions adjacent to them in labelled precursor solutions, showed maxima in DNA synthesis at 4-7 hours and RNA synthesis at 6-9 hours after the worms were cut. The head-forming blastema has higher uptakes for both DNA and RNA precursors than does the tail-forming blastema. The maximum for protein synthesis occured 24-27 hours after cutting the worms, again with the greater uptake in the head-forming blastema.

In interpreting the results of Kohl and Flickinger's chloramphenicol experiments, it should be noted that chloramphenicol, a broad-spectrum cytostatic agent, has the effect of stopping cell growth. It is not known to specifically to inhibit DNA synthesis so the latter's conclusions regarding a gradient of DNA synthesis as being Child's morphogenetic gradient, are pure speculation and are not supported by their data. McWhinnie (1955) obtained very much the same result - obliteration of head-frequency gradient - by the use of the mitotic spindle poison, colchicine. Blastema formation, but not eyespot differentiation, was inhibited. Also, Kanatani (1958) using demecolchicine (colcemide) in D. gonocephala found bipolar head formation. None of the above effects was observed in animals treated after cutting, indicating that

the crucial polarity determining events occur within the first 24 hours after cutting. Thus it would appear that the relationship between time to produce sufficient cell numbers and time for synthesis and transport or diffusion of hormones may be the crucial determinants of the head-frequency gradient. Such a view would not find it too difficult to explain the relation of axial polarity to electrical field strength demonstrated by Marsh and Beams (1952) and the volume resistivity data of Marsh (1962). Their results could be explained on the basis of an electrophoretic action on the hormones. The data of Coward (1964) are also not inconsistent with this hypothesis. It should prove interesting to find if Lender's hormone(s) moves in an electrophoretic field, and in which direction; especially in view of the findings by Lenicque and Lundblad (1966), of an electronegative nucleo-protein fraction present in regenerating hydrae, which promotes the number and growth of tentacles in regenerating hydrae. Shapira, Coleman and Castellani (1966) find that during the first 18 hours post-cut, the purine : pyrimidine ratios of head and neck segments of D. dorotocephala are different. Also, the ratios of RNA/LMW purines and pyrimidines are different. This might have some relation to polarity control.

4 The Rhabdoids

The rhabdoids (rhabdites) are protein in nature and

serve the function of keeping the planarian covered with a continuously renewable "fluid cuticle" which allows ciliary activity but protects the animal against abrasions, etc. (Jennings, 1957). Cutting the animal stimulates the massive release of these rhabdites, which rapidly hydrate, forming a getatinous clot-like envelope over the wound surface.

5. Factors Affecting Regeneration

An examination of the literature on planarian regeneration shows the following variables to have been considered:

(1) cephalo-caudal level of anterior and posterior cuts (transverse pieces).

(2) medio-lateral level of cut(s) (longitudinal and sagital pieces).

(3) size of piece

(4) extensiveness of wound surface(s)

(5) size of animal

(6) age of animal

(7) nutritional status of animal

(8) temperature.

Obviously, an experiment to determine the effects of any one variable must control the others. Unfortunately, in much of the early work this was not done. For the sake of brevity I shall cite only the results of the well controlled experiments.

(1) Cephalo-caudal level of anterior and posterior cuts:

As we have seen in section C 2 a., from the work of Child (1911b), Sivickis (1932), Watanabe (1935a) and Abeloos (1930), the level of the anterior cut surface is of crucial importance to the head-frequency of groups 2, 3 and 4. Similarly the level of the posterior wound surface determines the tail-frequency of these groups (Brøndsted 1939, 1942a).

(2) Medio-lateral level of cut(s): Brøndsted (1946, 1947[1]) and Brøndsted and Brøndsted (1952) have shown that the head-frequency gradient extends not only along the cephalo-caudal axis, but along the medio-lateral axis as well, i.e. the gradient is two dimensional. The medio-lateral gradient has a maximum along the midline and decreases towards either side (for Bdellocephala, Dendrocoelum and Dugesia lugubris.)

(3) Size of piece: Child (1911b) cut decapitated specimens of D. dorotocephala into eighths or sixteenths and found that head-frequency and survival of the eighths pieces was better than that of sixteenth pieces from the same level. Watanabe (1935a) confirmed this finding, comparing one eighth, one quarter, three-eighths and half pieces of D. dorotocephala all with their anterior surfaces cut from the same level, at several levels. Abeloos (1930, 1932), however, found that for D. gonocephala, the rate of regeneration (time of eye appearance) the amount of new tissue, and the head-frequency are all independent of the size of the piece. Buchanan's data

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1. Cited, Brøndsted (1955).

(1933) for Phagocata gracilis are in agreement with Abeloos for the anterior half of the animal, but agree with Child and Watanabe for the posterior half of the animal. Watanabe (1935b) showed that larger pieces of D. tigrina have a higher head-frequency than do smaller pieces. Raven and Mighorst (1948) showed that in D. lugubris size of piece has no effect on the rate and degree of regeneration. Brøndsted and Brøndsted (1954) confirmed the latter's findings and found that their conclusions are also valid for Polycelis nigra, Dendrocoelum lacteum and Bdellocephala punctata. Fedecka-Bruner (1961) has shown that for regeneration of the copulatory apparatus, the size of the piece is of major importance, and is more important than the cephalo-caudal position of the piece. Thus the importance of size of piece on regeneration ability depends on the species and the organ of interest. One other very interesting point with regard to size of piece and head-or tail-frequency is that if, say a quarter or eighth piece of planarian (D. dorotocephala or D. tigrina) is, after a delay of 6 hours (tail-frequency) or 36 hours (head-frequency) cut into an eighth or sixteenth piece, the tail- and head-frequencies (respectively) will not correspond to those of that level, but will be maximal (Child and Watanabe, 1935; Watanabe, 1935b), i.e. the tail-and head-frequency gradients have changed during the process of regeneration. These find-

ings demonstrate that morphallaxis can begin very shortly after the cut.

(4) Extensiveness of wound surface(s): Zeleny (1917) noted that simultaneous regeneration from other parts of the body does not retard regeneration. Brøndsted and Brøndsted (1954) confirmed this observation for Bdellocephala punctata. A transverse-cut animal, and another such animal but with two long lateral cuts as well (ratio of wound areas ~1:7) both regenerated heads at the same rate. However, for small pieces of planarians, if the wound surface is sufficiently extensive the piece will die. Cuts repeated within a short space of time, unless they have the effect of providing a morphallactic advantage, decrease the rate of subsequent regenerations.

(5) and (6) Size and age of animal: The size of an animal is a function of its age, past regeneration history and nutritional state. The data of Abeloos (1930) for D. gonocephala fed three times a week are consistent with a growth rate of $\frac{dm}{dt} = 0.88$ mg/day from hatching to full grown and a plot of $\frac{1}{m}\frac{dm}{dt}$ is also consistent with a straight line of constant decrease in relative growth rate with increasing age or size. An animal which is cut and then regenerates is physiologically (with respect to regeneration) identical to another animal of the same size[1]. A starved animal will

1. This is especially so if a small piece is cut off and allowed to regenerate so that the proportion of new tissue to old tissue will be large.

shrink and appear to be young again, but although it will be somewhat rejuvenated after it has been fed again, i.e. physiologically younger than it was when it was larger, its growth rate will not be the same as that of a young animal of that size. Abeloos (1927, 1930) found that small young specimens of D. gonocephala regenerated heads more rapidly than did larger older specimens (6 mm, 3.5d; 11 mm, 4d; 16 mm, 5d; 18 mm, 6d.) and that the number of abnormal regenerates increased with animal size. The former finding was confirmed by Sivickis (1933)[1] and by Scharoff (1934)[1] for Dendrocoelum lacteum. Scharoff also noted, however, that the probability of a very young animal regenerating increases over the first few months (1d, 7%; 20d, 25%; 30d, 55%; 60d, 60%).

(7) Nutritional status: The effects of starvation on the respiratory and metabolic rates have been discussed in section C 3b. In short, after an initial drop the respiratory rate rises upon prolonged starvation. Child (1911a), Sivickis (1933[1], 1934[1]) and Brøndsted (1953) all report a decrease in regeneration ability (Child - degree; Brøndsted - rate) for the many species studied. However, in all cases these authors were comparing starved and unstarved animals which were originally the same size. Abeloos (1930) compares starved down and then refed with fed growing young animals of the same size at the time of comparison and finds no effect of prolonged

1. Cited, Brøndsted (1955).

starvation on D. gonocephala with respect to speed of eye formation, this is affected mainly by the size of the decapitated planarian. However, if the starved animal is refed before decapitation, its growth rate will be faster than that of starved unfed animals and eye formation will be more rapid. Also, more regeneration abnormalities are found in the starved and unfed animals. Thus starved animals in a starved condition do not regenerate as well as starved animals which have been recently fed, or well-fed animals.

(8) Temperature: Child (1911a), Abeloos (1930) and Dubois (1949) found that decreases below the upper range of temperatures consistent with survival, increase the time necessary for regeneration (D. dorotocephala, D. gonocephala and D. lugubris).

6 Effect of Radiation on Planaria

Bardeen and Baetjer (1904) subjected regenerating planarians (D. tigrina and D. lugubris) to fractionated exposures (doses not stated) to 'medium soft' X-rays (10-20 min per day for 12-18 days), and found that, apart from wound closure no regeneration takes place, the animals dying 25-30 days after the first exposure. They did not describe the external changes prior to death except to say that the degenerative process began at the head and proceeded caudally. They observed that the roentgen rays exert a "powerful inhibitive effect upon cell reproduction in planarians . . .

(which) may be entirely stopped by sufficient exposure." The observation of a latent period in the action of X-rays, known to clinicians of that time, was noted. Thus the observation that the effects of radiation upon organisms are due "primarily to its action on cells capable of reproductive activity", soon to be formulated as the law of Bergonié and Tribondeau (1906), had already been made in this report. Histological examination of uncut animals at various times after the last exposure "showed no marked alterations in the muscular, nervous and intestinal apparatus. The cutaneous epithelium seemed to be normal except for a few areas where the cells were shorter and broader than usual. The cilia of the ciliated cells were intact." However, the cells of the testis showed no mitoses, and showed signs of degeneration. They also noted that "the rays have effect not so much upon tissue differentiation . . . (i.e. eye formation) . . . as upon cell-reproduction. The spreading out of the surface epithelium so as to cover a cut surface, whereby columnar epithelium becomes transformed into pavement epithelium also indicates this. Death in exposed specimens may possibly be due to a necessity on the part of the organism for a certain amount of cell-reproduction." After this brief but comprehensive report, one might have expected a surge of interest in planaria as a tool for studying radiation effects but for 20 years thereafter very little was done. Schaper (1904) using radium,

obtained results similar to those of Bardeen and Baeter (1904).

Curtis (1928) and his school hoped to use radiation as a new technique in the investigation of the nature of the "formative cells" and their role in regeneration. X-ray doses of 2500-3500r to P. maculata (D. tigrina) and P, agilis (D. dorotocephala) which have high regenerative powers and many formative cells, rendered them incapable of regeneration and led eventually (~30 days later) to death (Curtis, 1936). Lower doses allowed slow and/or imperfect regeneration (with much less new tissue) and some survivors had completely recovered and could regenerate normally 60-70 days after the irradiation. Sections of irradiated worms showed decreasing numbers of neoblasts with increasing doses, and at the "heaviest exposures these cells were so far eliminated that there seemed to be none remaining in many of the specimens" (q.v. Figure 5 in Curtis, 1936).

Weigand (1930) irradiating Polycelis nigra, Planaria torva and P. lugubris, with radium (exposures from 15 min to 16 hours) found that all the irradiated pieces were able to complete the wound healing phase of regeneration, but the amount of new tissue and the rate and degree of regeneration diminished with increasing exposure. Cutting the worms 1-3 days after irradiation resulted in progressively greater injury than in those cut immediately post irradiation. Planaria torva and Polycelis nigra were very similar in their responses, while P. lugubris showed somewhat more resistance

in that it formed more tissue, was less liable to deformation, and was less sensitive to post-irradiation delay in cutting. Cutting the worms from 1½ to 9 days pre-irradiation showed that the blastema became more resistant to the irradiation as it grew older, i.e. as it differentiated and as cellularity increased. Feeding was shown not to affect the pattern of postirradiation changes (in the first 21 days, after which all survivors were fixed). The histological study showed that cellular changes (pycnosis and absence of mitoses) could be recognized as early as 2 hours post-irradiation. The highly differentiated cells of the gut, sense organs, and nervous tissue and musculature remained unchanged until affected by the final deformation and degeneration of the piece.

Strandskov (1937) giving X-ray doses of 86-864 r to D. dorotocephala, also observed that the degree of regeneration decreases as the time (0-6 days) between irradiation and cutting increases.

Curtis (1936) reviewing the effects of radiation on regeneration, concluded that, "cellular changes . . . seem to precede the organismal changes appearing as the failure of regeneration and the degeneration that follows." He also pointed out the basic interpretative drawback (of that time) of the radiation and regeneration experiments with planarians. "The weakness of such results . . . lies in the disputed

origin of the formative cells, in the difficulty of recognizing formative cells as such, and in the fact that individual planarians, after becoming wholly incapable of regeneration as a result of such irradiation, cannot be maintained alive and in an otherwise normal state for a long period. If the formative cells are a persistent embryonic stock, necessary for other cell replacements (my italics) and for sexual reproduction as well as regeneration, it would perhaps be too much to expect that the individual thus deprived of these cells could live out a normal span of life, whatever that may be for a given planarian."

Many authors, e.g. Dubois (1949), Chandebois (1963b), have described the post-irradiation (5-10 kr) syndrome in many planarian species. This syndrome is much the same for all planarians.

After a variable period of delay (a function of dose and temperature) the first radio-lesions appear. The epidermis recedes in the sensory areas of the auricles which roll in on themselves and on the anterior edge. A scar membrane is formed and pigment (from degenerating rhabdites) condenses behind it. The same pattern occurs ventrally. The head forms a cup shape, concave ventrally, and is progressively resorbed. The anterior edge of the planarian comes to appear like the scar found after cutting a planarian, bordered by a collar of necroses, Dubois' 'collier de nécroses', which represents the

accumulation of pigment gathered by the resorbed epidermis. The same process occurs shortly afterwards, in the tail, and later still, around the pharynx. The ventral turning in of the edges may progress until the animal is in the shape of a trough (D. gonocephala types). The body of the planarian becomes distended and ruptures develop in the epidermis, from which a whitish mass of cytolysed cells pours forth. Sometimes these ruptures are sealed with scar tissue, but new ruptures develop. Sooner or later a major opening occurs resulting in the lysis of the animal within a few hours. The survival time is highly variable but, unless animals are kept for a "long time"[1] in the laboratory, animals collected in the spring and summer resist longer than those collected in the autumn, and winter.[2] The length of this delay is thought to be determined by the nutritional state of the animal - pre-irradiation starved animals remaining intact longer (approx. 12 days).

Chandebois (1965b) found that D. subtentaculata decapitated immediately post irradiation (8-10 kr) regenerate new heads. Moreover, no matter what the level of the cut, scarring and blastema formation occurred at a slightly slower rate than the unirradiated controls, the eyes appearing a day or

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1. ~ 2 months
2. q.v. Chapter V.

two later than in the controls. However, the growth of the regenerate is blocked (ceases) about 10-18 days after the cut. Tails can also be regenerated, but they regress again contemporaneously with the cephalic region. The irradiation does not inhibit fission[1] (asexual reproduction) either, but animals which do divide seldom complete their regeneration. If the cut is made 24 hours post-irradiation, the regenerate is smaller than that of the animal cut immediately post-irradiation. However, if the cut is made 48 hours post-irradiation, no regeneration takes place. Dubois (1949) found that D. lugubris cut 24 hours after 5 kr forms an abortive blastema which regresses from the 4th day on. This abortive regeneration can be seen to be due to the mobilization and differentiation of sterile (in the sense of loss of reproductive integrity) neoblasts. As they do not continue to divide, but progressively degenerate post-irradiation, there ceases to be a supply of cells from which to build the regenerate. Worn cells are not replaced and the resorption of the blastema follows. Thus Chandebois' conclusion that the differentiation of the cells of the blastema does not necessarily require prior mitosis, is not at variance with Dubois' findings that the blastema is built exclusively of neoblasts.[2] Chandebois has not proven her hypothesis that the abortive blastema is due to local tissue dedifferentiation. In the

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1. q.v. Chapter XIII.

2. q.v. Chapter XII. The differences in their results are due to species differences.

48 hour post-irradiation cut D. subtentaculata Chandebois claims that the inhibition of regeneration is not due to lack of undifferentiated material, but its loss of ability to differentiate. That irradiated neoblasts which are in the process of degeneration should with time lose their ability to differentiate, should also not prove surprising. Chandebois (1965a) suggests that the order of the appearance of the radio-lesions (cephalic region, ventrum mediodorsum, etc.) may indicate differences in renewal rates of these tissues.

In another paper, Chandebois (1965b) reports that the lower the X-ray dose given, the longer post-irradiation one must wait before cutting in order to produce an abortive regeneration. 350 r would not prevent regeneration, but a wait of 11 days after 525 r would produce an abortive regeneration. However, the 525 r animals would survive and eventually recuperate. From 700 r up, the delay decreases from 4-2 days but none of the worms survive.

Attempting to take advantage of the polyploid biotypes of D. lugubris to test for radiosensitivity differences with ploidy, Benazzi (1962b) studied the effects of hard and soft X- and γ- radiation on oöcytes and embryos at various stages of their development. Doses of 1000-1300 r (200 kV X-rays) were tolerated by the ovum during the first hours after deposition (first cleavage division occurs 4-6 hours

post-deposition) but some individuals did not hatch, stopping somewhere in the middle of their development. 1500-2000 r is lethal to the ovum (even if given in the first few days post-deposition) which can undergo cleavage and blastomere organization but can develop no further. If the embryo has already developed a definite pharynx and eyes before the irradiation, it may survive. 3000-6000 r to the adult resulted in death 3-5 weeks later. Benazzi also noted the radiosensitivity of the neoblasts. However, Benazzi was not able to quantitate his results sufficiently to be able to compare the vari-ploid biotypes. Benazzi (1963b) was able to show that sperm irradiated with doses of up to 70,000 r contained lethal mutations such that the embryos of the diploid synaptic and amphimictic biotype were unable to hatch, whereas those of the polyploid (triploid) asynaptic, amictic biotype were totally unaffected, thus demonstrating that the activating power of the sperm is not affected by such doses.

Le Moigne (1965c, 1966) studied the effects of 60 kV X-rays on the development of P. nigra embryos. Doses below 500 r did not destroy all the neoblasts and regeneration occurred in all animals. Doses between 750-3000 r had varying effects depending on the developmental stage of the embryo. Eggs and Stages 1 and 2 never survived. Development never went beyond the blastomere and transitory organ stage.

Stage 3 and 4 were at a critical period, with 50% developing abnormally and with abnormal neoblasts (the other 50% dying). None were able to regenerate. The last three stages (5 to 7) were not prevented from hatching. However, they were also depleted of neoblasts and none were able to regenerate. Kratochwil (1962) showed that regeneration can be completed, in spite of large doses of irradiation, provided that such doses are given at a time when the blastema is differentiating (and thus not in need of many more neoblasts).

Regarding Chandebois' insistance on the syncitial nature of the regenerating parenchyma, it is of interest that Rohlich (1966) shows that OsO_4 fixation (that of Chandebois) produces different structures (vacuolation) than does glutaraldehyde fixation (microvilli membranes) of planarian photoreceptors. Rohlich suggests that the OsO_4 fixative is the one which produces artifact as the vaculolated structure is of lower energy than the membrane system of the microvilli. Also, the OsO_4 fixative does not produce this artifact in well developed (normal) adult planarian photoreceptors; only in the regenerating and in the degenerating photoreceptors does OsO_4 fixation yield this artifact. Thus the cellular nature (fixed parenchymal cells) of the parenchyma and of the blastema demonstrated by Pedersen (1961a), Ishii (1966), Le Moigne et al. (1965) and others, can be taken to be the true struc-

ture. The aneuploid metaphases observed in D. subtentaculata by Chandebois (1965a) using Prenant's (1922) method of "contact prints" might well indicate that her material is polyploid. Le Moigne (1962) also notes aneuploid spermatogonia, oögonia and neoblasts in the triploid Polycelis tenuis. In no case does he find neoblasts with less than the diploid chromosome constitution. Benazzi (1966) gives evidence suggesting that in polyploid chimeras local differences in chromosome constitution may occur. Considering Le Moigne's (1963) description of embryonic development, it is not difficult to see how such local differences could be established, especially if the chimerism occurred during an early phase of development.

	Neoblasts	Oöcytes	Synaptic	Spermatocytes	Sperm	Zygote
A	2n	4 bivalents	+	2n	n	n ♀ + n ♂ → 2n Amphigonic 2n
B	3n	12 bivalents	+	2n	n	3 n ♀ + n ♂ → elimination of n ♂ → pseudogamic 3 n ♀
C	3n	12 univalents	–	2n	n	3 n ♀ + n ♂ → elimination of n ♂ → pseudogamic 3 n ♀
D	4n	16 univalents	–	2n	n	4 n ♀ + n ♂ → elimination of n ♂ → pseudogamic 4 n ♀

Table I - 3

Reproductive Processes of the D. lugubris Polyploid Series

Table I-4

Proportion of Neoblasts in Cell Cycle

	0.5% mitosis in body yields % cells in cycle	5% mitosis in blastema yields % cells in cycle
P + M + A + T = 7% of cycle	7%	70%
M + A + T = 4.4% of cycle	11%	100%

Chapter II

The Problem Stated

Chapter II

The Problem Stated

As we have seen in the introduction, the *in vitro* clonal studies of exponentially growing mammalian cells of normal and malignant varieties show (with few exceptions) a common type of survival curve (with respect to loss of reproductive integrity after irradiation by medium to hard X- or γ-rays) with a surprisingly narrow range of values of the curve defining parameters. Similar results are obtained in the *in vivo* assay for the loss of reproductive integrity of exponentially growing malignant tumour cells (Hewitt and Wilson, 1959), and have been deduced for the exponentially growing stem cells of the *Vicia* root meristem (Hall, Lajtha and Oliver, 1962). The nature of the cells (spleen colony-forming-units) of Till and McCulloch (1961) is not very well understood, but they too exhibit this common postirradiation survival pattern.

Withers (1967) and later Emery (1967) were able to determine, for an organized tissue of non-exponentially growing cells (mouse dermis), the slope of the exponential portion of the assumed survival curve by a statistical cloning method based on the Poisson distribution. However, they were unable to define the survival curve as the extrapolation number, or even its range, remained indeterminate. Kember (1967) has

used a similar method to study rat growth cartilage and obtained similar results - i.e. unable to define E.

The success of Hall et al. (1962) in the deduction of a survival curve for the meristematic cells of Vicia was the first reported quantitative explanation of the effects of radiation on the growth of an organized tissue in terms of a cellular mechanism, the loss of reproductive integrity. The crucial link between the cellular radiation response and the postirradiation survival of a highly differentiated multicellular organism has been assumed by most authors, but to date there has been no report of a successful study either quantitatively linking the two in terms of a testable model, or by the elimination of such a testable model(s), determining what other factors intervene between the assumed independence of each cell with respect to its loss of reproductive integrity and the subsequent death or survival of the organism.

The first object of this thesis is to examine the factors influencing the postirradiation survival of a simple multicellular animal, the planarian, with a view to:

(1) constructing a simple testable model from which the survival curve for the reproductive integrity of the animal's stem cells can be deduced from (related to) the survival characteristics of the organism.

and (2) testing the model to find what other factors, if any, must be taken into account and quantitated before organismal survival can be explained quantitatively at the cell-

ular level.

We have already noted the attempts of early radiobiologists and proponents of the target theory to implicate the nucleus and more specifically the chromosomal DNA as the "target" or sensitive site for radiation induced loss of cellular reproductive integrity. The only experiments using autopolyploids or allopolyploids to test this hypothesis have been performed on plants /where the results for higher plants, e.g. Chrysanthemum, have been totally at variance with those for single celled organisms, e.g. Saccharomyces cerevisiae/ with contradictory results, and just recently in animals, with the in vitro Chinese hamster cell (Bedford and Hall, 1967).[1] The other animal polyploid experiments, e.g. Révész and Norman (1960), and Silini and Hornsey (1962) have been based on aneuploid material or, e.g. Hamilton (1967), Metalli and Ballardin (1962), Atwood et al. (1956), on developing eggs and embryos - rather special cases.

As euploid polyploidy is not uncommon amongst the Planariidae, the second object of this thesis is to compare the radiosensitivities of diploid, triploid and if possible, tetraploid animals; and in the light of the results of the first object of this thesis, to determine the importance of haploid karyogramic multiplicity on the radiosensitivity of an animal cell in vivo.

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1. This material is in fact aneuploid but the 4n+2 hypertetraploid was derived from the 2n+1 hyperdiploid by the use of a mitotic spindle poison, thus making it highly likely that the entire chromosome complement has been doubled.

Chapter III

The Experimental Material

Chapter III

The Experimental Material

A Species Considered

The species considered for this study (those which it was attempted to breed in the laboratory) were:

Dugesia lugubris	(O. Schmidt)	biotype A	(diploid, 2n=8)
" "	"	"	B (triploid, 3n=12)
" "	"	"	C (triploid, 3n=12)
" "	"	"	D (tetraploid, 4n=16)
" "	"	"	E (diploid, 2n=8)
" "	"	"	F (diploid, 2n=6)
" etrusca	(Benazzi)		(diploid, 2n=16 and hypotetraploid aneuploids)
" ilvana	"		(diploid, 2n=16)
" tigrina	"		(" 2n=8)
" benazzii	(Lepori)		(polyploid)
Polycelis nigra	(O. F. Müller)		(diploid, 2n=16)
" "	"		(triploid, 3n=24)
" hepta	(Hansen-Melander, Melander and Reynoldson)		(diploid, 2n=14) (" 2n=12)
" tenuis	(Ijima)		

Polycelis felina (diploid?, 2n=28)

Dendrocoelum lacteum (O. F. Müller) (diploid, 2n=14)

B Source of Animals

1 The Shropshire Meres

The Shropshire meres of Ellesmere (D. lugubris B, P. nigra, P. tenuis, and Dendrocoelum lacteum), Colemere (D. lugubris B, P. tenuis, Dendrocoelum lacteum and D. tigrina), and Crosemere (D. lugubris B, and P. tenuis) are highly productive and 300 to 500 planarians can be collected by two people in the course of a day. Table III-1 shows the relative proportions of the various species obtained from each mere. Frequent chromosome analysis confirmed that the D. lugubris collected in these meres were of biotype B.

2 A Spring near Hastings

A spring swelling up from an underground source and running along side a country lane (Ord. Surv. TQ 7916) off Kent Street, Sedlescomb, near Hastings, provided large numbers, seasonally, of Polycelis felina. At one time 8,000 animals were collected in a space of 2 hours without any signs that the original population had been decreased.

3 The "Orto Botanico", Pisa, Italy

By the kind permission of Professor M. Benazzi of the Department of Zoology and Comparative Anatomy of the University of Pisa, and of Dott. F. Garbari, the curator of the Botanical

Garden of the Department of Botany of the University of Pisa, I was allowed to collect several hundred specimens of D. lugubris A from an artificial pond in the Botanical Garden. This is a pure population of diploid D. lugubris and has been confirmed as such by chromosome analysis.

4 The laboratory of Professor M. Benazzi

Through the generosity of Professor M. Benazzi and Mme. Dott. G. Benazzi-Lentati, 67 specimens of D. lugubris D were provided me. This biotype is extremely rare in nature and consequently has been developed and bred in the laboratory by the following series of crosses:

2n amphimictic oöcytes

3n asynaptic oöcytes

Toblino ♀ x Roma ♂

↓

F_1(2n) 4 bivalents in the oöcytes

(2n) (3n)

Toblino ♀ x Roma ♂

↓

F_1(2n) 4 bivalents in the oöcytes

x

↓

$\frac{2}{250}$ F_2 (4n)

↓

with 16 univalents in the oöcytes,
16 chromosomes in the neoblasts,
pseudogamic, asynaptic, amictic

F_3 as above $2F_2$

↓

F_4 and succeeding generations as above $2F_2$

In addition, Professor Benazzi gave me 10 specimens each of D. etrusca (Benazzi) (from Toscana), D. ilvana (Benazzi) (from Isola d'Elba), D. benazzii (Lepori) (polyploid, from Sardegna), and P. nigra (polyploid, from Pavia). The latter two species did not grow in my laboratory and were lost. A second population of 67 specimens of D. etrusca was given to me after the first population had succumbed to a lytic disease. This population was aneuploid.

5 The laboratory of Professor P. Pasquini

Through the generosity of Professor Pasquini of the Department of Zoology and Comparative Anatomy of the University of Rome, some 200 specimens of D. lugubris C were provided me. They did not breed well in our laboratory.

6 The laboratory of Dr. Y. Melander

Dr. Melander was kind enough to send me several specimens of clonal populations (obtained from one animal by repeated cutting and regeneration) of D. lugubris A (Lago Maggiore, Italy) and D. lugubris F (Värnamo, Sweden), as well as some natural specimens of D. lugubris E (Lake Ivösjön, Sweden). The latter two species did not grow well and were lost. The former species survives but grows too slowly to allow one to obtain the number of animals necessary for radiation-survival experiments.

C Collection Techniques

1 Rock sampling

This method, which consists of picking up stones, slates

or bricks lying in the shallow water of productive lakes and streams and removing with a scalpel or a jet of water those planarians which had been sheltering themselves on the underside, is probably the simplest and most productive means of planarian collection from densely populated lakes.

2 Bait setting

This method, described by Reynoldson (1960), can be productive but is more complicated and is recommended only when a simpler method is unavailable. Strips of beef liver (Reynoldson recommends freshly cut slices of lumbricus) are places on a weighted hook and thrown into the water. After a minute or two ($\lesssim$ 5 minutes) the line is pulled in and planarians which are feeding on the bait are washed off. The bait is then recast.

3 Leaf sampling

During the summer and autumn months, a favourite resting place for planarians inhabiting deciduous lakes or ponds is on the underside of lilly pads or leaves. To collect the planarians one need only pick up the leaves and wash them off into a jar with a jet of water from a squeeze bottle.

4 Bottom sampling

If the planarians are on a very fine silt bottom, they are easily collected by means of a wide bore pipette (25 ml, with tip cut off) with a large rubber bulb attached to one end. This method was particularly suitable for the stream

near Hastings, where 8,000 animals were collected in 2 hours.

5 Transportation

If the planarians must be kept in the collection flask(s) for more than four or five hours after collection, a thermos flask should be used. Care must be taken to leave an airspace so that the animals do not exhaust the water's oxygen supply. As freshly collected specimens are usually hungry and are placed in a relatively small volume of water, it is advisable, if the water can be changed within 24 hours, to add a few medium sized pieces (~1x1x5 cms.) of liver or of some other food so that they do not devour each other. If planarians are being sent from one laboratory to another, it is advisable to feed them 24 hours before packing them into their bottles or thermos flasks. If only small quantities are being transported, then it is sufficient to put them in plastic bottles surrounded with damp cotton wool in a biscuit tin.

D Maintenance

1 Containers

Three types of containers were used. Animals which must be kept isolated, either for experimental reasons, or to prevent sexual reproduction between members of a clone, were placed in disposable 13 ml. or 50 ml. crystal polystyrene tubes (Camlab) with perforated polythene caps. All other animals were kept in glass bottles (squat type - Solmedia), ~30-50 adults per 400 ml. bottle.

2 Water

All animals were kept in mains water, i.e. water brought into the laboratory directly from the mains via iron and lead plumbing. In many other laboratories, planarians are kept in tap water, but as our laboratory water supply travels quite long distances in copper pipes, the water became sufficiently rich in Cu^{++} to become toxic (lethal) to planarians, this special provision was necessary. As all planarians were kept in this mains water and the Cu^{++}-rich tap water of our laboratory was not used in connection with the planarians, the terms mains water and tap water will be used interchangeably.

3 Food

Animals were fed once or twice a week on rat or beef liver, cut into small pieces ($< 1cm^3$), the water being changed and waste products removed the following day. Tubes and bottles were replaced as soon as they showed signs of becoming dirty. Occasionally the adults are fed tubifex worms (Tubifex tubifex), although this is used mainly for freshly hatched young.

4 Temperature

All animals were kept in a $20^{o}C$ incubator, the air temperature of which varied by less than $\pm 1^{o}C$. Temperature fluctuations of the water in the incubator was much less than that ($< \pm 0.2^{o}C$).

5 Population Growth Rates

The population growth rates of the fissiparous D. etrusca (clone 1) and D. ilvana are shown in figure III-1 and that of D. etrusca (2nd population - q.v. p. 98) in figure 2. The rate of increase for the pseudogamic tetraploid D. lugubris D is shown in figure III-3. The rates of increase of the cocoon laying species are deceptively high in that after hatching, there can be a high mortality rate until animals reach adult size. Amongst the triploid D. lugubris B this is mainly due to cannibalism.

E Species used for the Radiation Experiments

The species used for the radiation studies are those which either grew well in our laboratory:

Dugesia lugubris A (2n)
" " B (3n)
" " D (4n)
" etrusca (2n, 4n-)
Polycelis tenuis-hepta (2n)

or those which are sufficiently abundant in nature that sufficient numbers can be collected for an experiment:

Dugesia lugubris B
Polycelis tenuis-hepta
" felina

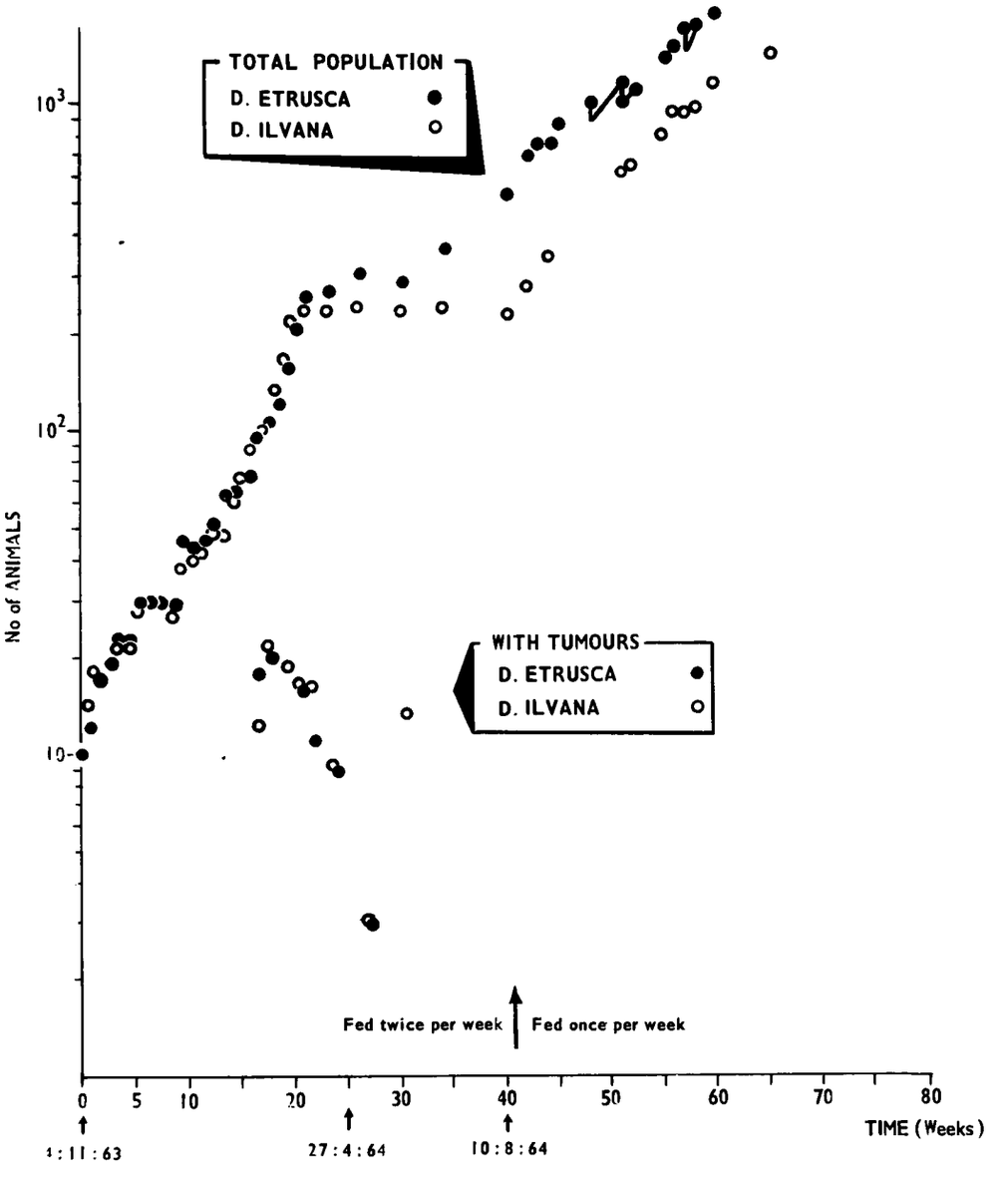

Figure III-1

Population growth curves of D. etrusca (diploid clone 1) and D. ilvana

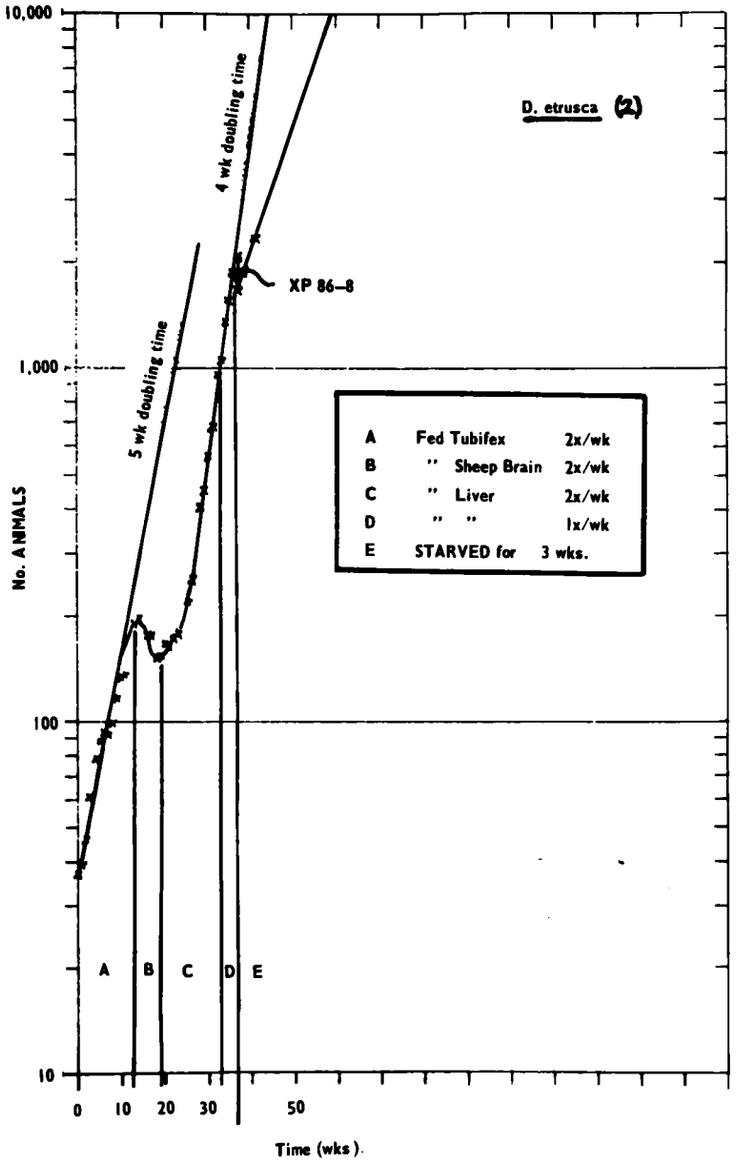

Figure III-2

Population growth curve of D. etrusca (o ula-
tion 2, aneu loid)

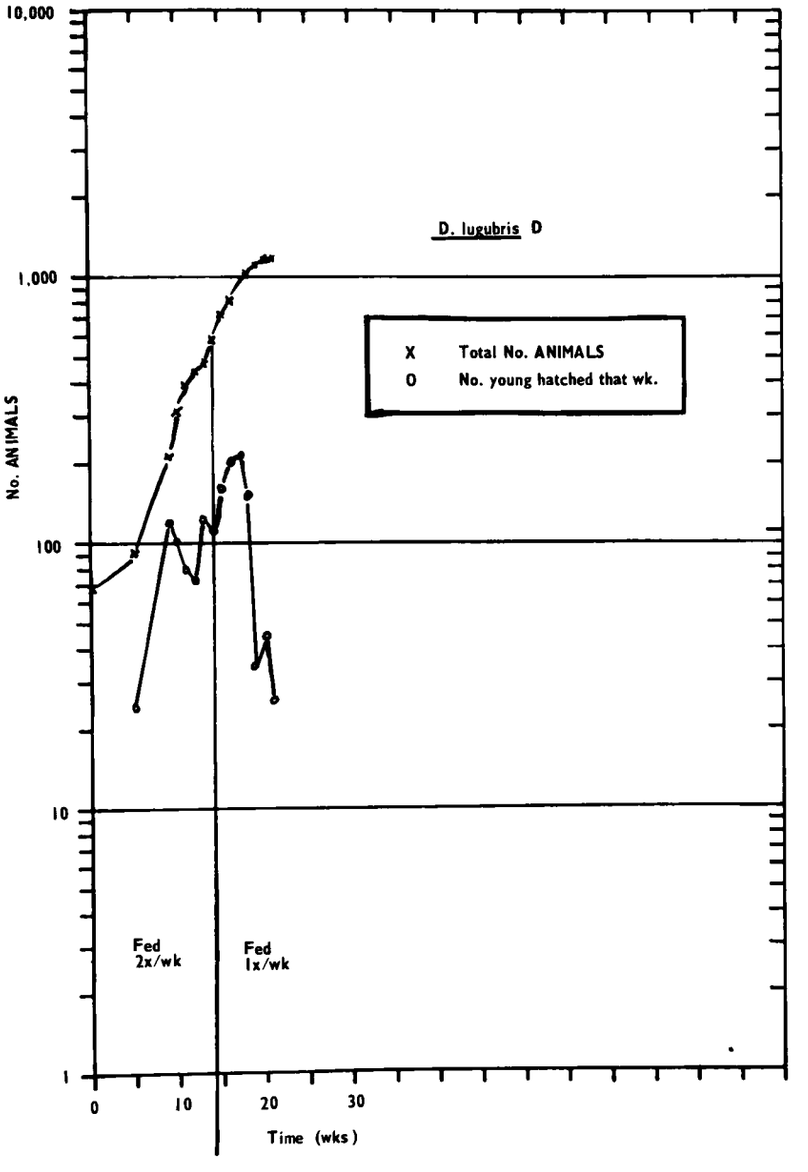

Figure III-4

Population growth curve of D. lugubris D (tetraploid)

Table III-1

Results of Planarian Collections in Shropshire

Date	Site	Total	D. lugubris B	Polycelis	Dendrocoelum
1/6/65	The Mere and Colemere	607	257	100	250
20/6/65	The Mere	576	360	88	128
	Colemere	177	49	6	122
24/10/65	The Mere	861	746	33	82
29/1/66	The Mere	721	461	207	53
	Colemere	112	17	84	11
13/3/66	The Mere	264	138	114	12
	Colemere	615	102	457	56
14/5/66	The Mere	65	40	25	-
	Colemere	387	150	213	24
28/5/66	Crosemere	1322	115	1200	7
31/5/66	Colemere	1227	446	673	108
31/6/66	The Mere	795	460	210	125
1/4/67	The Mere	956	599	332	25
	Colemere	277	84	157	36
	Total:	8,962	4,024	3,899	1,039
	%	100	45	43.5	11.5

Chapter IV

Irradiation Methods

Chapter IV

Irradiation Methods

A X-rays

All X-ray experiments were irradiated on a "Resomax 300". The physical parameters were: 300 kVp, 19.5 mA, filtration 1.0 mmAl, H.V.L. 1.9 mmCu. The Resomax was fitted with an internal integrating dose meter which registered the dose given (at the position of the monitor) on a digital counter. When the preset dose had been given, a relay was tripped automatically closing the shutter and turning down the kilovoltage. Thus dosimetry was reduced to a matter of testing the uniformity of the field (flatness and depth) and relating the dose at the point of interest to the dose measured by the internal monitor.

As it was considered desirable to keep the planarians in water during the irradiation, a waterproof tissue equivalent container was necessary to restrain the planarians to a relatively small volume of water surrounded by water equivalent scattering material. The scattering material serves to flatten the radiation field. The obvious material from which to fabricate such a container, would be one of the plastics. However, in view of the report of Paterson (1942) to the

effect that irradiated Perspex (Polymethylmethacrylate) and Distrene (an I.C.I. product) give off substances toxic to cells grown in vitro, it was first attempted to test the toxicity of irradiated polystyrene on Hela cells (on the assumption that cells grown in vitro should be more easily reached by and perhaps be more sensitive to radiation induced pyrogens than would aquatic multicellular organisms). Four polystyrene tubes were sterilized in methyl alcohol and dried under a U.V. lamp. Two tubes acted as controls while the other two were irradiated to a dose of 10^4 rads.* Each tube was then inoculated with 10^4 Hela cells in 3 ml. and incubated at 37°C for 4 days. At the end of this time the contents of each tube were counted on an electronic cell counter (Celloscope). The control tubes each had 5×10^4 cells while the irradiated tubes had 5×10^4 and 8×10^4 cells, respectively. Thus it is unlikely that crystal polystyrene produces radiopyrogens of sufficient toxicity to have any effect on planarians, and should therefore be a suitable material for the fabrication of irradiation chambers.

In figure IV-1 we see a drawing of the crystal polystyrene irradiation dishes, and figure IV-2 shows the irradiation setup (jig 29). The dose rate to the top dish is ~195 rads*/min (1.74 rads*/monitor unit), and the field has a 20 cm diameter at 40 cm F.S.D. Dosimetry was

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* rads in soft tissue

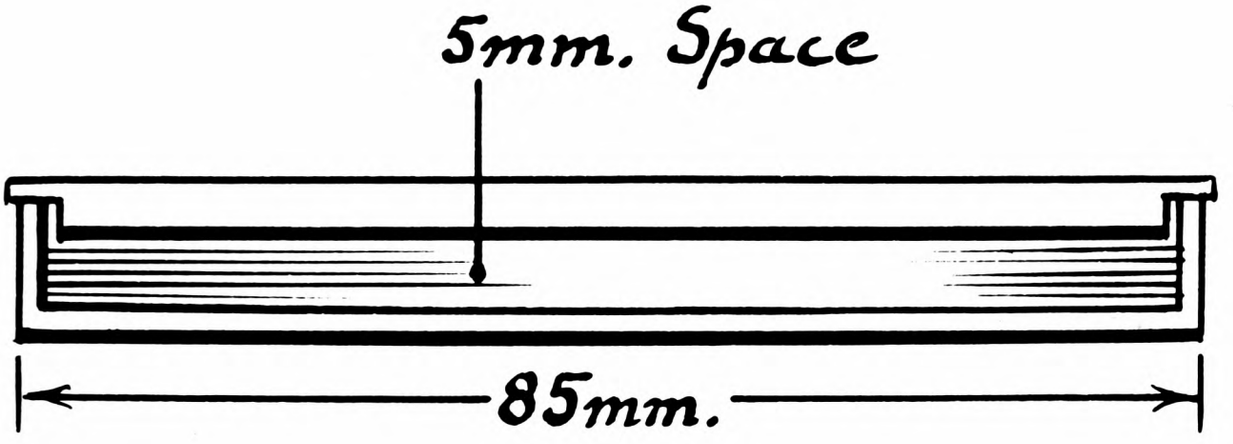

Figure IV-1

Drawing of one of the crystal polystyrene dishes used for X-irradiations

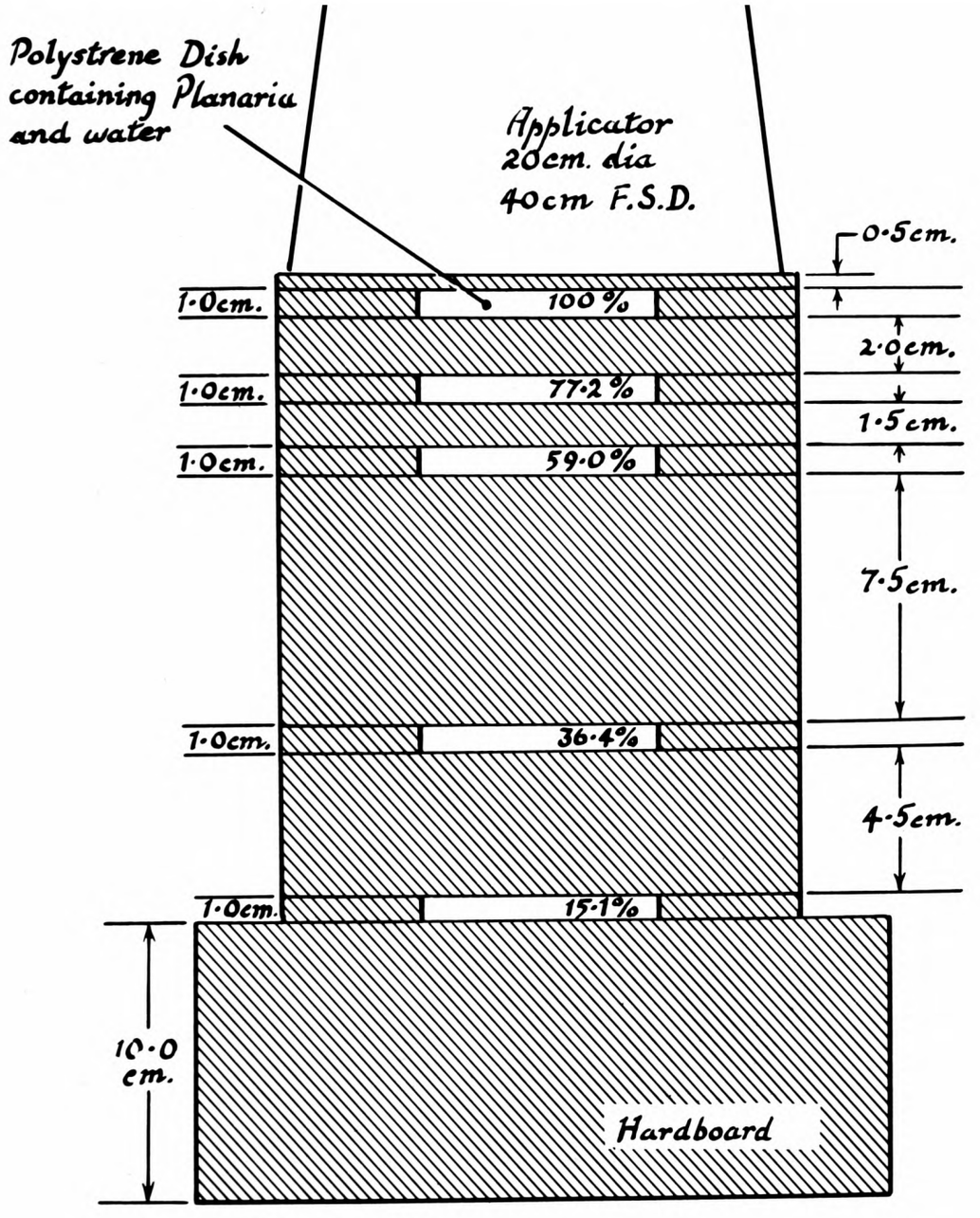

Figure IV-2

The irradiation setup for X-irradiations

carried out with a Baldwin Farmer ionization chamber and with LiF. For the top dish the flatness of the field was tested by placing LiF samples at 4 points 3.5 cm from the centre (along 2 diameters perpendicular to each other) and a fifth sample in the centre of the dish. The probable error for beam flatness was 1.3%, for field depth (± 2.5 mm water) was 1.3%, and for X-ray output was 1%, giving a total probable error of ± 2.1%.

Animals to be irradiated were placed, up to 20 or 30 per dish, in the irradiation dishes and irradiated as shown in Figure IV-2. If a spacing of doses other than that obtainable by the spacing of dishes in the jig was required, the bottom four dishes were replaced by bolus-filled irradiation dishes and the appropriate radiation dose was delivered to the animals in the top irradiation dish.

After irradiation, unless otherwise stated, the animals were removed from the irradiation dishes and placed in individual 13 ml crystal polystyrene tubes (q.v. p.100), filled with tap water. The tubes were then capped with perforated polythene caps and placed in the 20°C incubator (q.v. p.101). Head-frequency determination experiments were examined daily while LD_{50} determination experiments were examined thrice weekly for the duration of the experiment - usually 100-150 days postirradiation.

B Strontium - 90 β^- rays

In order to test the existence of a relationship between the number of reproductively intact neoblasts and the LD_{50} of the animal, while leaving the total mass of differentiated tissue a constant, it was desired to shield parts of the planarian while irradiation-sterilizing the rest. Stéphan-Dubois and her school have done many such shielding experiments (e.g. Dubois, 1949) using 60 kV X-rays and a lead shield, and Fedecka-Bruner (1964) has sterilized the dorsal neoblasts and testis using 5 kV X-rays (H.V.L. 0.2-0.3 mm water) while the ventral neoblasts were not sterilized as could be seen from the regeneration which followed. However, for 300 kV X-rays, if one considers the radiation dose to the supposedly shielded regions, side scatter alone must be of the order of 5% of the total dose given to the sterilized portion, and as the dose distribution is not uniform, such an irradiation procedure would not suffice for an experiment which has as its object, the determination of the effects of radiation on the neoblast population. Thus a means of irradiating parts of a planarian, while shielding with a sharp dose cut-off boundary and eliminating side scatter as a serious contributor to the dose received in the shielded region, was desired.

$^{90}Sr/^{90}Y$ β^- rays are of sufficiently low energy that they can be totally stopped by 55 thou' of stainless steel. Figure IV-3 shows the plan of the plastic "sandwiches" which

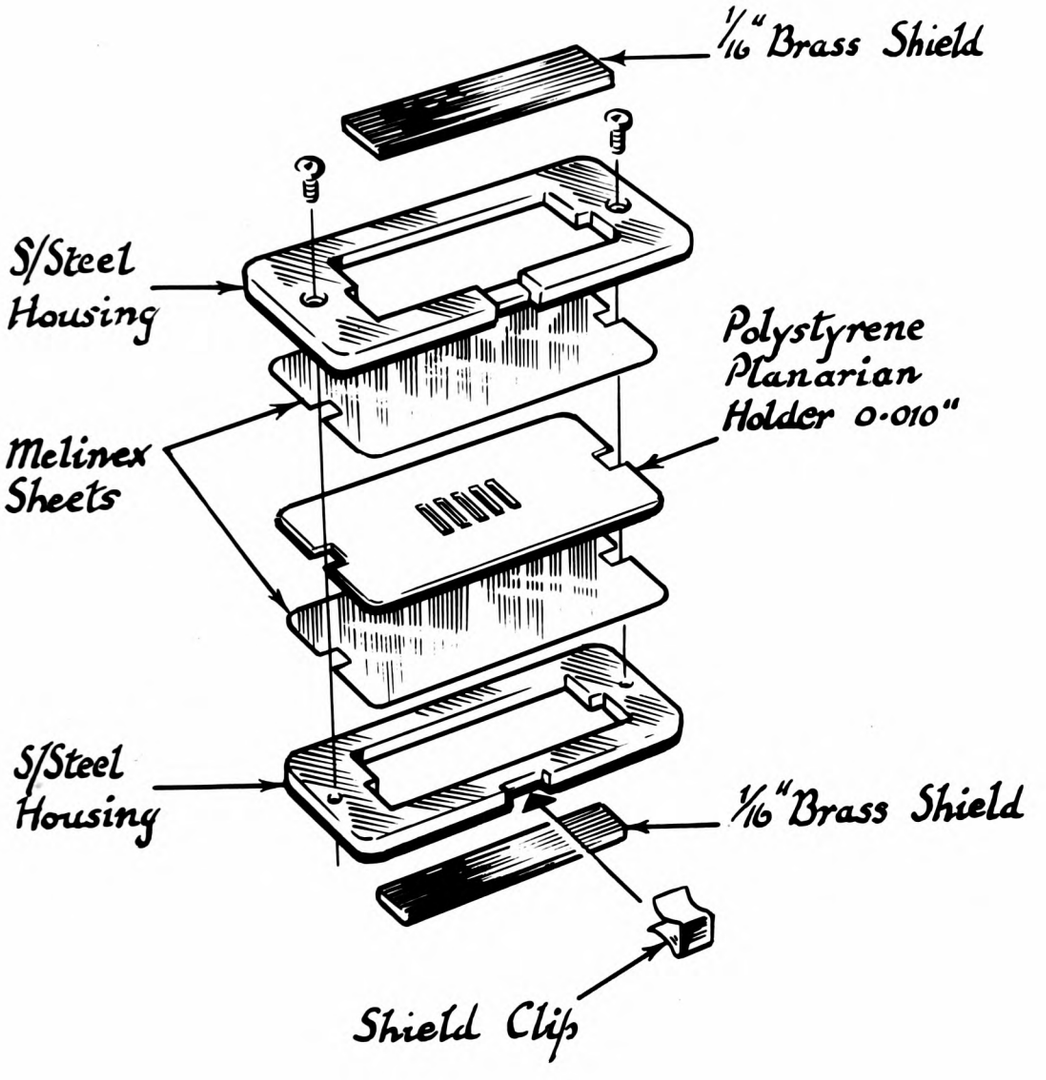

Figure IV-3

Plan of the "plastic sandwich" for holding planarians during $^{90}Sr\beta^-$ irradiation

The position of the holes determines the shielded proportion of the animals.

held the planarians and the shields during the irradiation, and in figure IV-4 we see a drawing of the irradiation device.

The isodose curves for the "window" of irradiation are seen in figure IV-5; and figures IV-7 and IV-6 show, respectively, the dose drop off along the short axis of the isodose elipse with and without the shields in place. The dose delivered was equal to the stated dose within less than ± 19.3 rad, or 1%, whichever was the greater.

For a given experiment, the size of the animal (and thus the appropriate sized cavity to hold that animal), the proportion of the body length to be shielded (defining the position of the cavity), and the nominal dose to be given to the shielded portion (before applying the shield and supralethally irradiating the exposed remainder), were assigned the indices i, j, k respectively. The first two indeces defined the appropriate irradiation jig to be used, and the order of irradiation was determined by the sequential appearance of the numbers ijk in a table of random numbers. As the whole experiment usually took several days to irradiate, this randomization was of great importance. If a given dose group (ijk) was to have more than one group of 5 animals, then the second set of irradiations would be similarly, but separately, randomized such that all values of ijk for the first block of irradiation had to be filled before

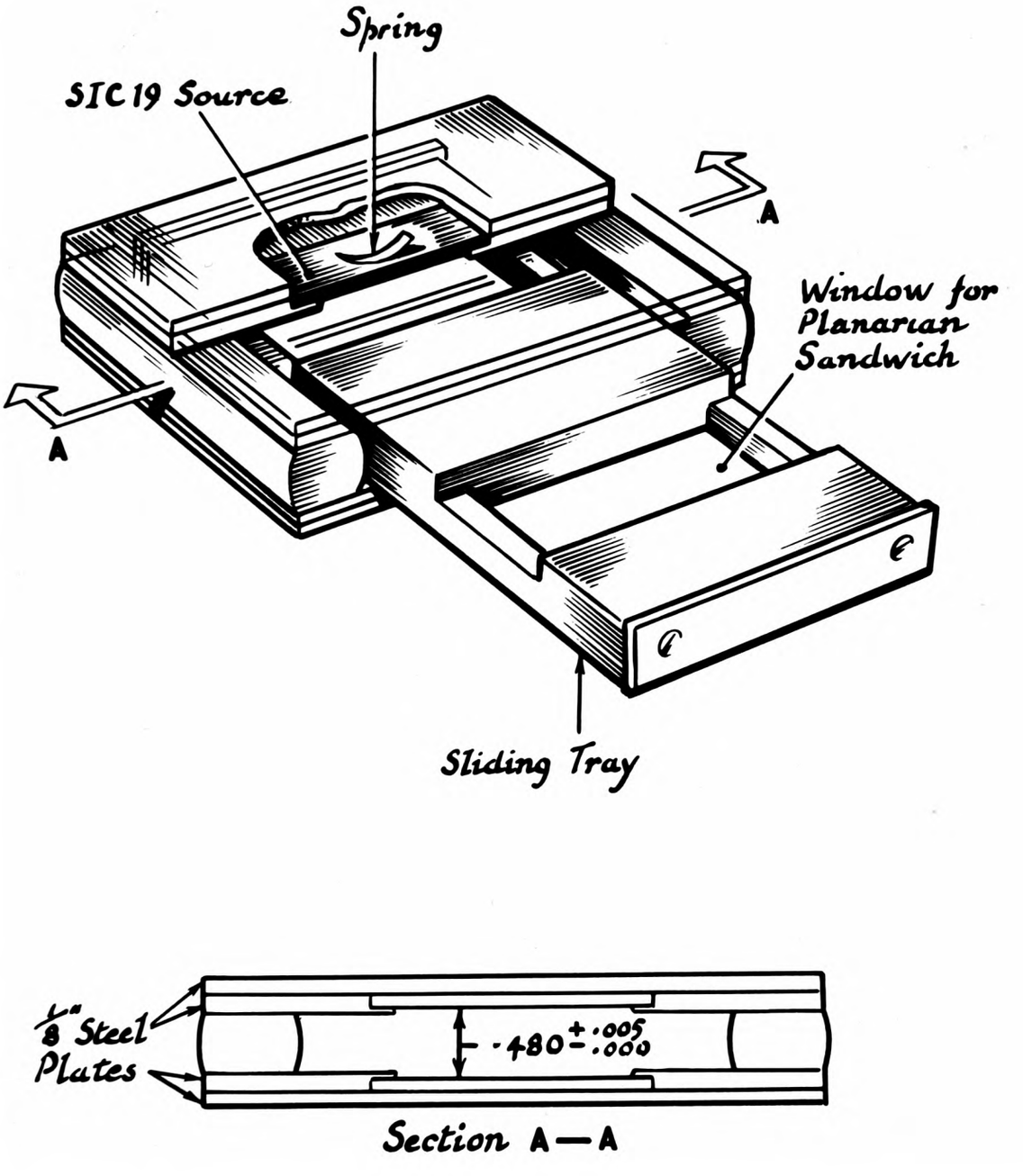

Figure IV-4

The ^{90}Sr irradiator

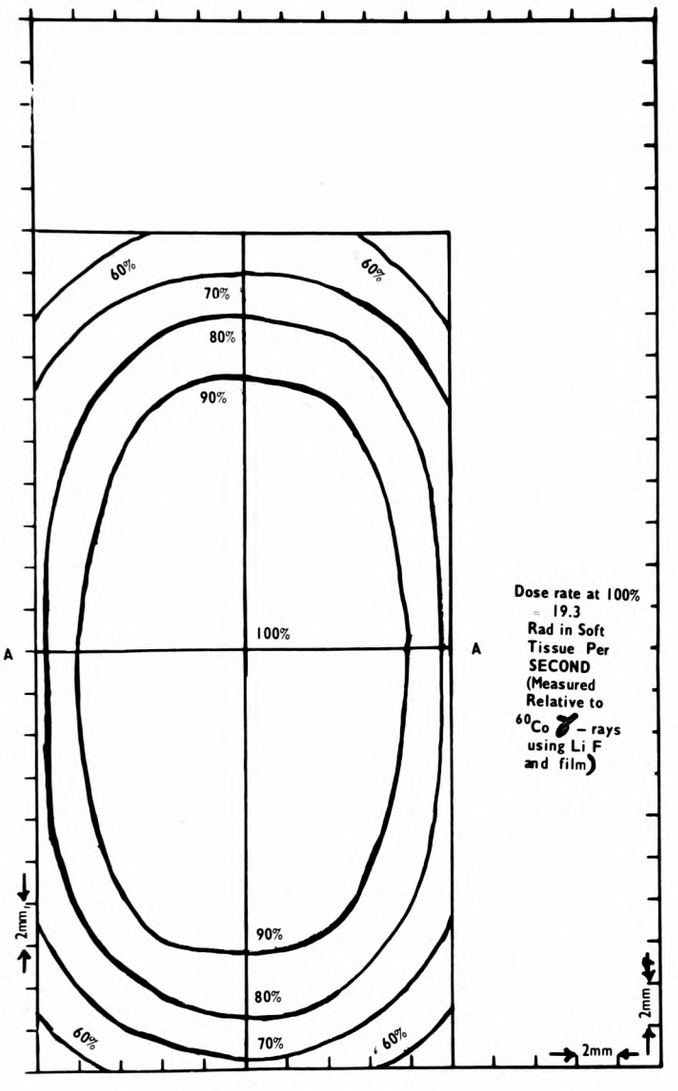

Figure IV-5

Isodose curves for ^{90}Sr irradiation

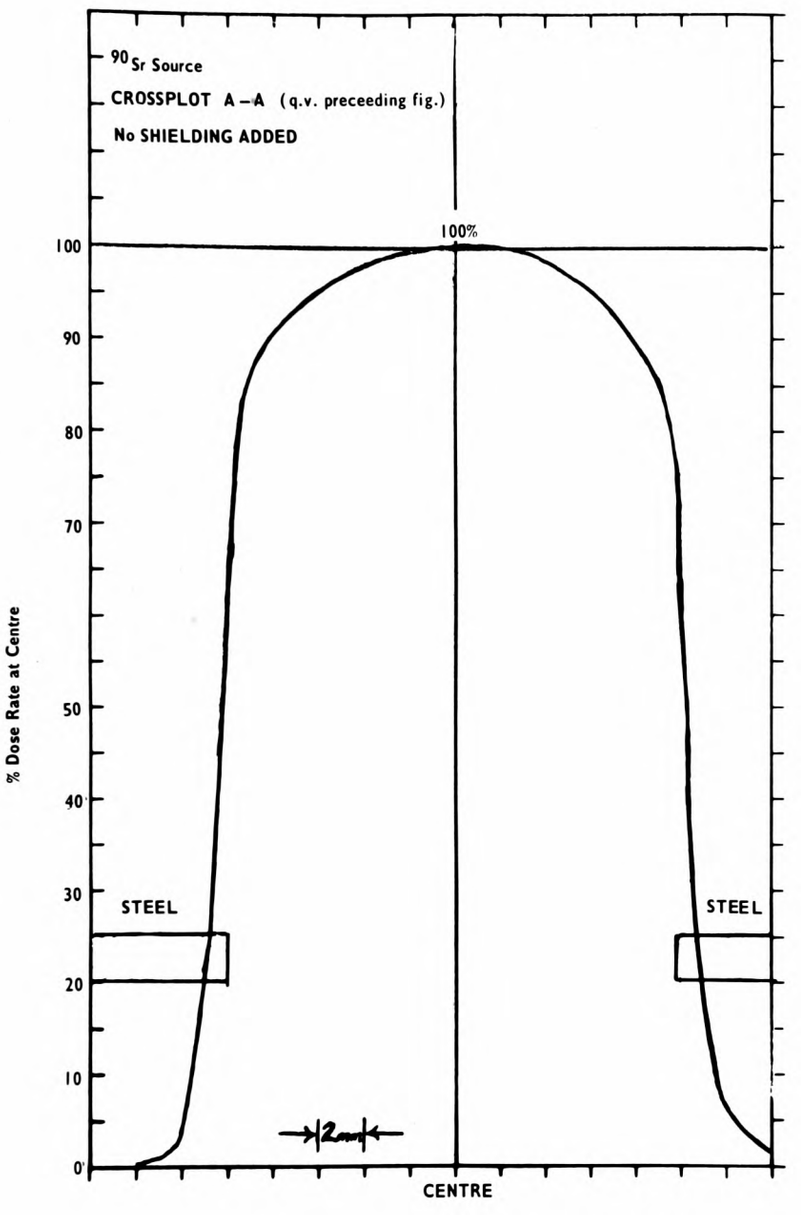

Figure IV-6

Dose distribution along short axis of isodose ellipse

No Shielding.

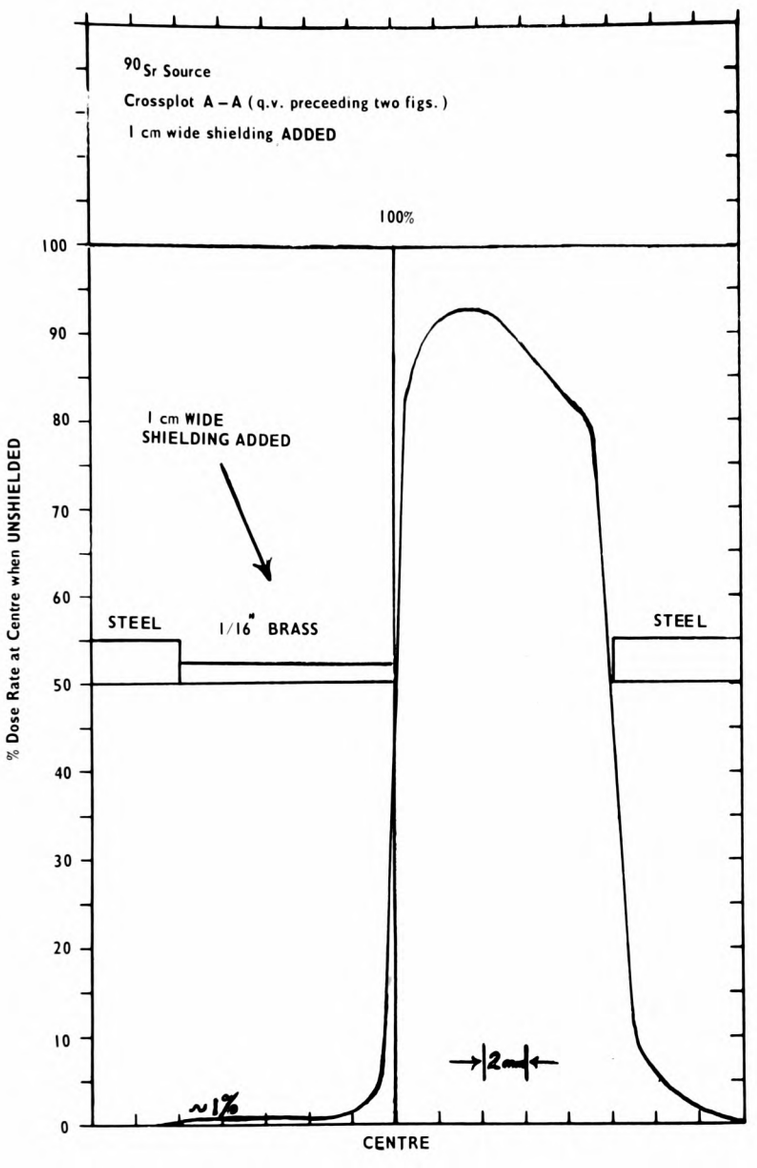

Figure IV-7

Dose distribution along short axis of isodose ellipse

Shields in place.

an ijk from the second block could be assigned a position in the queue. The reason for this separate randomization was to prevent an unequal weighting of dose groups (in terms of the number of animals to be used to determine that point) from altering the randomization of the order in which groups were irradiated. Irradiations were carried out in a hood where an air temperature of 14-20°C was maintained.

Chapter V

Results of X-Ray Experiments

Chapter V

Results of X-ray Experiments

A. Definition of Endpoints

1 Criterion for Survival

All of the species used in the experiments reported below, possess well developed powers of regeneration; such that even a small piece of a planarian is capable of becoming a complete animal and growing to adult size again. Thus, if even a small piece (as small as 1% of the animal by volume) of an irradiated planarian remains intact (unlysed), the animal would still have survived the irradiation. As a demonstration of this, in a few cases, such small pieces were allowed to grow to adulthood.

Thus the criterion for mortality used in all experiments was that the entire animal be lysed with no piece left intact. Until the last piece had lysed, the animal was considered a survivor.

2 Selection of Endpoint Times

The time course of postirradiation mortality for a typical X-ray survival experiment is shown in figure V-1. In the dose range $LD_{50} \pm LD_{50}$, the graph of the proportion of animals surviving (ordinate) as a function of postirradiation time (abcissa) follows the same pattern. For the first 30 days postirradiation there is no change in % survivors (although necrotic processes may appear). Death (complete

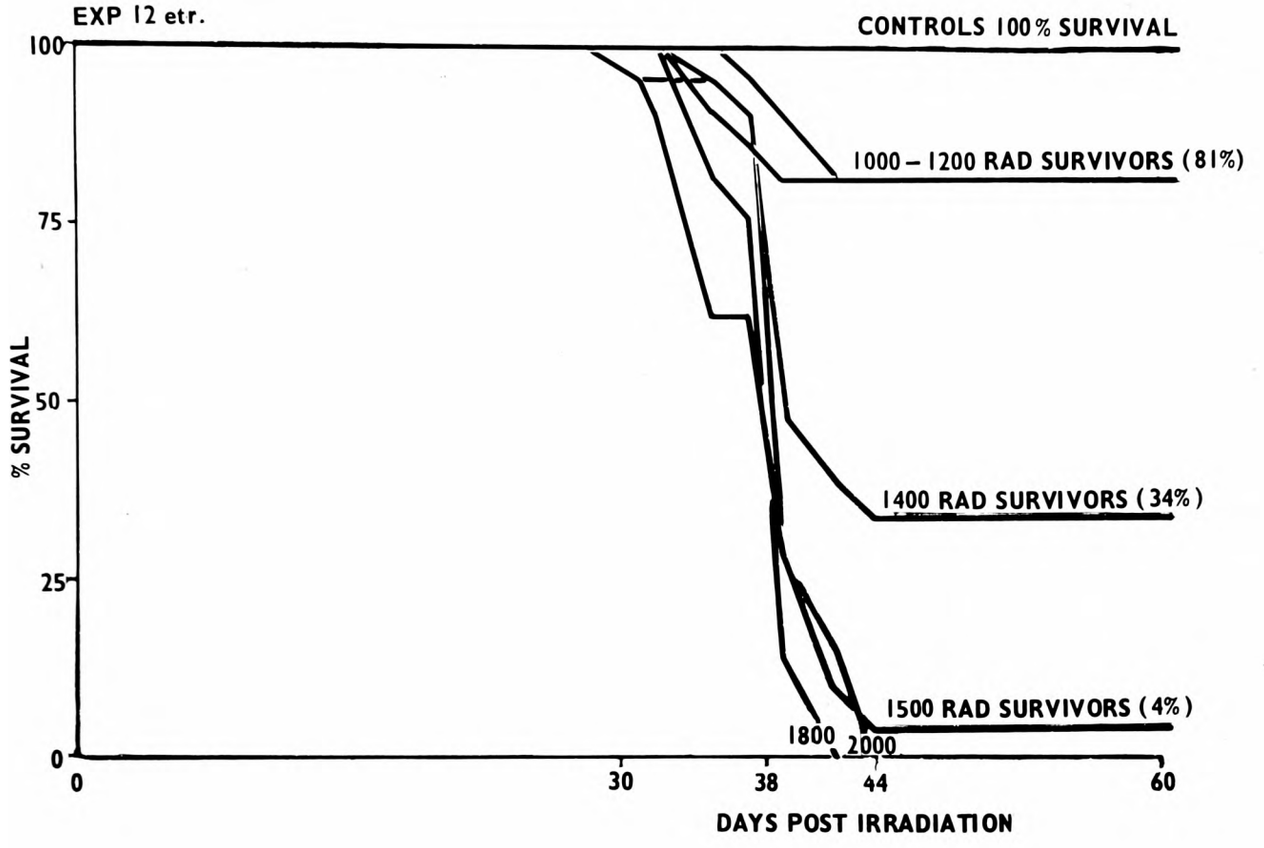

Figure V-1

The time course of postirradiation mortality - from a typical experiment

lysis) occurs between day 30 and day 45 (period of mortality). After day 45, % survivors remains constant. Animals were routinely examined for 100-150 days postirradiation to confirm the constancy of % survivors after day 45. In some cases, survivors were watched for nearly a year postirradiation before being discarded. Sixty day postirradiation survival was therefore taken as a general endpoint time. In those cases where the timing of the above pattern was delayed, an appropriate later endpoint time was chosen. Deviation from this pattern was found to occur only in the species Polycelis tenuis - hepta, in which case the period of mortality extended over a considerably longer period. Furthermore, the exact time of death for each animal was difficult to establish as this species encapsulates (figure V-2) in a thick sheath of mucus. Recovery from the radio-lesions occurs, or fails to occur, while the animal is encapsulated, and lysis is slow and progressive in this species.

B Estimation of Animal Survival Curve Parameters

For the endpoint time chosen, the planarian X-ray dose-survival curve is sigmoid and can therefore be fitted in probits by the Maximum Likelihood Method of Finney (1962). This procedure was computerized and the equation fitted was of the form: $y = mx + b$, i.e. probit $(P - C / 1 - C) = (1 / K)$ (Dose) $+ (-LD_{50} / K)$ where P is the observed mortal-

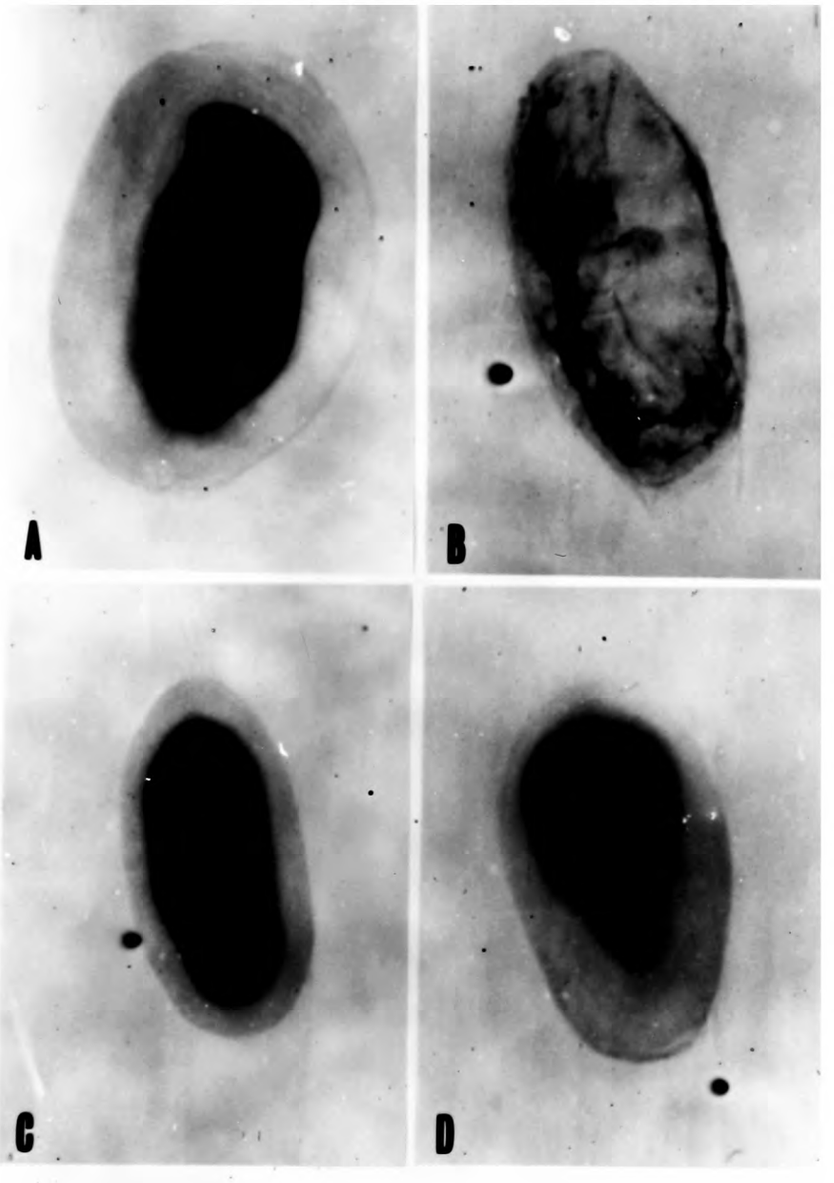

Figure V-2

Mucus capsules of Polycelis tenuis-hepta

a & c) planarian in capsule

b) empty capsule after recovered planarian has left

d) capsule with slowly lysing planarian in it.

ity probability, C is the natural mortality probability, K is the inverse slope of the probit line, and LD_{50} is the intercept of the probit line at 50% mortality. The argument of the probit operator, (P - C / 1 - C), is known as the Abbott kill, or Abbott's correction for natural mortality.

A sample print-out for one experiment is presented as figure V-3. The input consists of the doses given (including the control dose of zero), the number of animals at risk for each dose, and the number of animals affected (mortality) at each dose. The output consists of the two parameters of the probit line, the LD_{50} and K, and the natural mortality, C; together with their respective standard errors of fit, the total Chi-square and its degrees of freedom, the number of iterations performed[1], and the variance:covariance matrix for the fit.

The standard errors of fit may be slight overestimates, as they include the correction factor: Chi-square per degree of freedom to allow for the case where significant, but not systematic, deviations of the data from the fitted line exist. This is discussed by Finney (1962). In order to prevent underestimation of the standard errors, by this correction factor, for those cases where the Chi-square per degree of freedom

---

1. A minimum of three iterations were carried out, and more if necessary to reduce the parameter changes from the previous cycle to less than one standard error (S.E.) of each parameter, up to a maximum of nine iterations.

5.212α 1 2.235α 1 1.161α 1 4.560α 0 3.441α 0 4.097α 0 4.095α 0

270667 SET 1 PROBIT ANALYSIS PROBIT((P-C)/(1-C)) = (DOSE - LD50)/K

DOSE	NUMBER	DEAD	FRACTIONAL MORTALITY P EXPERIMENTAL	FITTED	(P-C)/(1-C)	WEIGHTED VARIANCE	EXPERIMENT NUMBER
0.000α-99	20	0	0.000α-99	0.000α-99	0.000α-99	0.000α-99	10701
1.250α 3	20	3	1.500α -1	2.032α -1	1.500α -1	3.497α -1	
1.400α 3	20	11	5.500α -1	5.050α -1	5.500α -1	1.619α -1	
1.500α 3	20	14	7.000α -1	7.172α -1	7.000α -1	2.907α -2	
1.600α 3	20	19	9.500α -1	8.721α -1	9.500α -1	1.088α 0	
1.800α 3	20	19	9.500α -1	9.881α -1	9.500α -1	2.466α 0	
2.000α 3	20	20	1.000α 0	9.996α -1	1.000α 0	2.556α -4	
2.400α 3	20	20	1.000α 0	1.000α 0	1.000α 0	1.617α-13	
2.500α 3	20	20	1.000α 0	1.000α 0	1.000α 0	0.000α-99	

*PARAMETER	1/PARAMETER 2	LD50	1.398α 3	S.E.	2.91α 1
	1/PARAMETER 2	INVERSE SLOPE K	1.780α 2	S.E.	3.50α 1
	PARAMETER 3	NATURAL MORTALITY C	0.000α-99	S.E.	2.23α -2

TOTAL CHISQUARE 4.095α 0 6 DEGREES OF FREEDOM 6 ITERATIONS

VARIANCE COVARIANCE MATRIX

1	2.57α 0		
2	-1.76α -5	1.22α -6	
3	-4.61α -5	2.73α -6	4.99α -4

Figure V-3

Sample rint-out for one experiment

falls below unity, this ratio was arbitrarily set at unity, as lower values would be fortuitous in data expected to fit a Gaussian distribution.

C Standardization of Experimental Routine

1 Effects of Pre- and Postirradiation Feeding

Planarians in their natural environment are subject to a variable food supply and can tolerate starvation for up to four or five months with no adverse effects other than shrinkage due to the catabolism of their own differentiated tissues (Rosenbaum and Rolon, 1960; Lender and Gabriel, 1961). As a planarian's metabolic state is a function of its food supply and the temperature of its environment (Hyman, 1951; Brøndsted, 1953), experiments to determine the effect of feeding on the following aspects of postirradiation survival were performed: survival, as measured by the survival curve parameters LD_{50} and K; mean time of death, t_m; and the natural mortality.

a. Postirradiation Feeding

i. D. etrusca

With postirradiation feeding the survival time of D. etrusca decreased by 30 - 40% (median time of death changes from 43 days to 27 days, mean time of death changes from 39 days to 25 days; $P < 0.001$. q.v. figures V-4 and 5 and tables V-1 and 2). The % survivors was also reduced, e.g. from 74% to 2.5% after 1000 rad ($P < 0.001$).

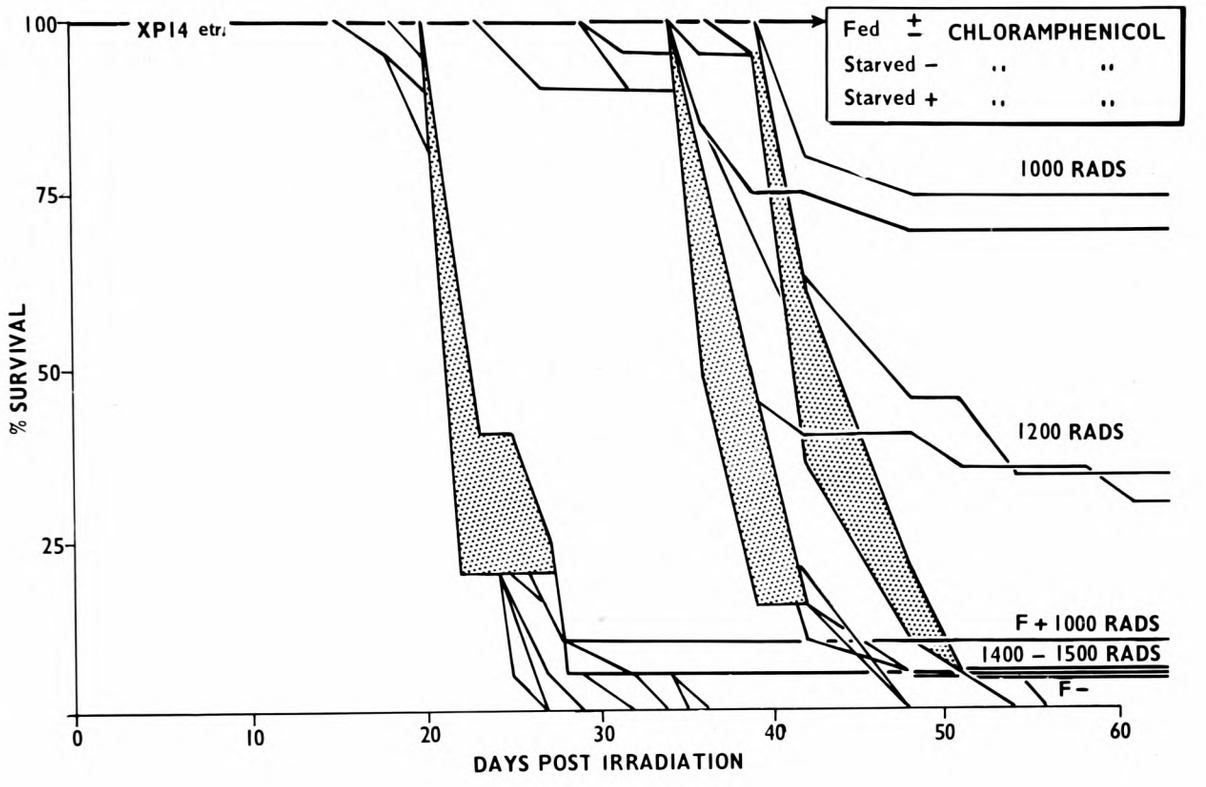

Figure V-4

Effect of postirradiation feeding, and/or antibiotics on survival of D. etrusca

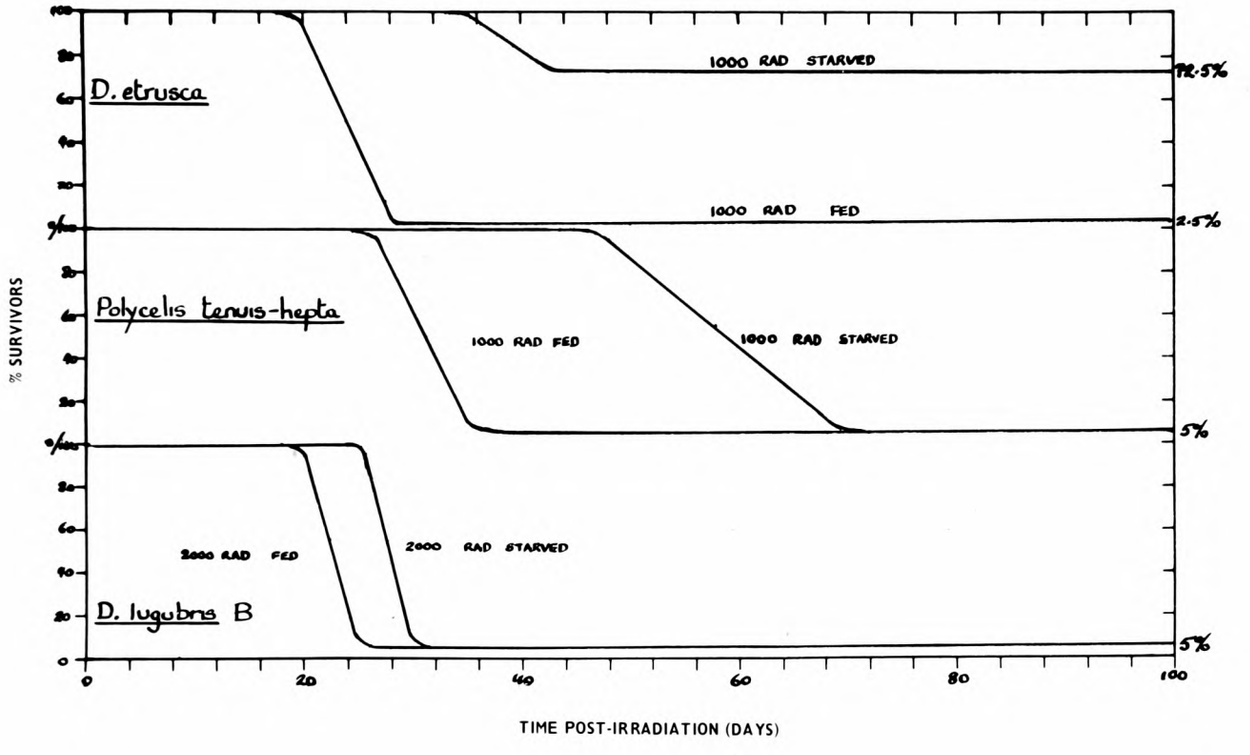

Figure V-5

Effect of postirradiation feeding on survival time

Thus postirradiation feeding is extremely deleterious to the survival of D. etrusca.

ii. D. Lugubris B

D. lugubris B shows a small decrease in survival time with postirradiation feeding ($0.05 > P > 0.01$). The mean time of death (after doses of the order of the LD_{50}) drops from 27.4 ± 0.9 days to 22.5 ± 1.3 days postirradiation. The slopes of the survival curves also differ significantly ($0.05 > P > 0.01$), becoming steeper after feeding. The LD_{50}, however, does not decrease significantly. Thus postirradiation feeding can be said to be slightly deleterious to the survival of D. lugubris B, in that the survival time was decreased and % survivors was decreased at doses somewhat greater than the LD_{50}.

iii. Polycelis tenuis-hepta

Polycelis tenuis-hepta survival curves show no significant changes in LD_{50} or slope after postirradiation feeding, if reasonable endpoint times are chosen for the two groups (60 days if fed, 120 days if starved); but the decrease in postirradiation survival time, from 58.4 ± 4.9 days to 30.6 ± 2.0 days, is highly significant ($P < 0.001$). Thus the only effect of postirradiation feeding on Polycelis tenuis-hepta is to alter the rate of mortality with time without affecting the absolute level of survival at any dose.

b. Preirradiation Feeding

The possibility of a deleterious effect of preirradia-

tion starvation was suggested by the results of some early experiments with D. etrusca. The LD_{50} for a population of animals irradiated after a 20 day period of preirradiation starvation was significantly lower than that for one irradiated after a 16 day starvation period ($0.05 > P > 0.01$). Even shorter preirradiation starvation periods appeared to produce a greater heterogeneity of response to the irradiation, as might be expected if the duration of the preirradiation starvation period was related to survival (LD_{50}). However, further experiments using shorter preirradiation starvation periods, showed a small but significant oscillation in LD_{50}; and attempts to repeat these observations in a second, nonclonal, population of D. etrusca failed to demonstrate any correlation between duration of preirradiation starvation (up to 20 days) and postirradiation survival (LD_{50}). The LD_{50} oscillation observed in the first population (clonal) and the constancy of LD_{50} observed in the second population (nonclonal) are shown in figure V-6. The probit line parameters and mean times of death for each of the starvation periods are given in table V-3.

The duration of the preirradiation starvation period was found to have no effect on the LD_{50} for D. lugubris B, nor could any effect be found for any of the other species studied (table V-3).

On the basis of the results of the pre- and postirradia-

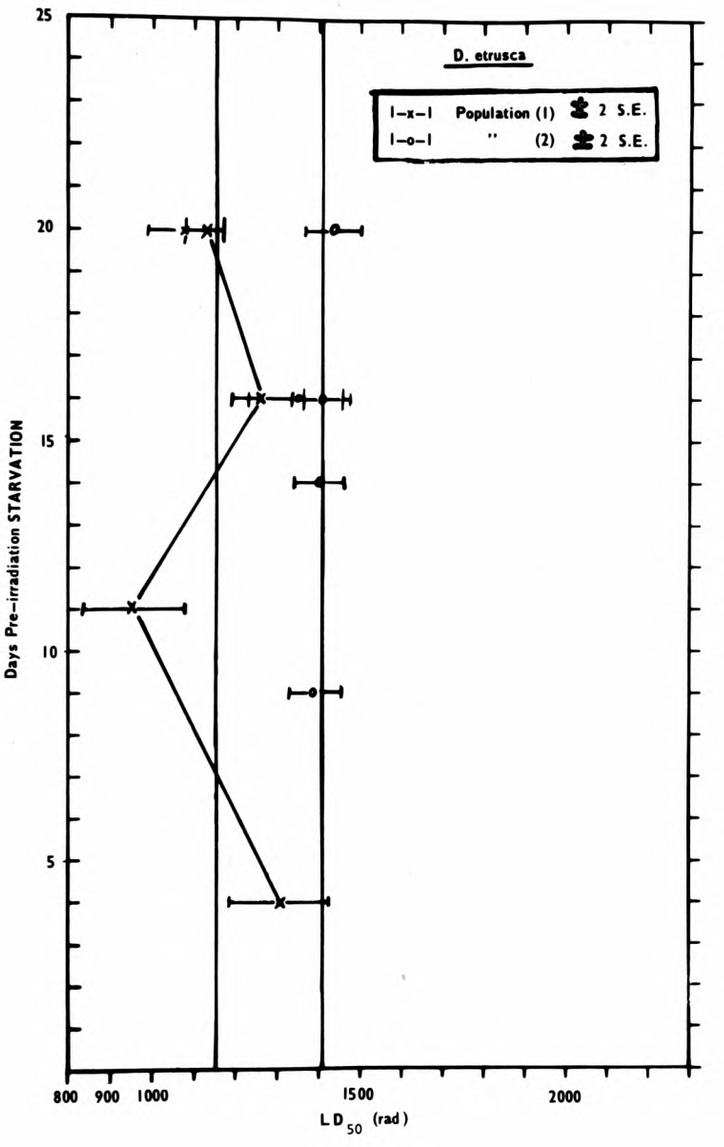

Figure V-6

Effect of duration of preirradiation starvation on LD_{50} of D. etrusca

tion starvation experiments, a routine of one or more weeks pre- and continuous (until well after the endpoint time) postirradiation starvation was followed.

2 Effects of Antibiotics

a. Chloramphenicol-induced Bacteriostasis vs. Penicillin + Streptomycin-induced Asepsis

In order to test the hypothesis that postirradiation death might be due in part to loss of ability to control commensal flora, the following two postirradiation treatments were performed:

(1) One test group was placed in tap water to which chloramphenicol had been added to a final concentration of 25 μg/ml. - a concentration shown by Henderson and Eakin (1959) to have no effect on planarian regeneration but sufficient to prevent growth of the flora commensal to the planarian (*D. dorotocephala*).

(2) Another group was cleared of commensal flora by the method of Ansevin and Buchsbaum (1961) - which is sufficient to provide aseptic explants for *in vitro* culture. The planarians were washed in two changes of a penicillin-streptomycin solution (2500 units of each/ml) followed by several sterile distilled water rinses and were then returned to tubes containing sterilized tap water.

Both treatments were then used in a comparison of the relative effects of the removal of commensal organisms by

penicillin + streptomycin, and of the bacteriostasis produced by chloramphenicol. The effect of the antibiotic treatments was to delay slightly (14-21%) the time of death of those destined to die; but the % survivors at each dose were not significantly different from untreated planarians. In both cases, no significant change was found in the parameters of the probit survival curves, but the increase in survival time was found to be highly significant ($P < 0.005$). The penicillin + streptomycin treatment yielded a greater increase in survival time than did the chloramphenicol treatment. This difference was significant ($0.05 > P > 0.01$) (q.v. table V-4).

In order to confirm that the antibiotics employed were effective in their control of organisms commensal to the planarians in our laboratory, the following experiment was performed:

b. Identification of Planarian Commensal Organisms Present in Lysates of Irradiated Planarians and Antibiotic Sensitivity Tests

i. Identification of Organisms

Five tubes, the contents of which are given in table V-5, were set up and their contents allowed to lyse. Aliquots from each tube were cultured in nutrient broth[1] at room temperature, and then plated for identification and sensit-

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1. The nutrient broth culture consisted of a 1 : 1 mixture of the aliquot to be tested and a sterile solution of Oxoid nutrient broth No.2 (CN67) at a concentration of 50 g/ml tap water.

ivity. The organisms found in each tube are listed in table V-5.

ii. Sensitivity to Antibiotics

Of the antibiotics tested (q.v. table V-6), only Colomycin, Kanamycin and Noxytiolin controlled all of the organisms.

Further cultures in nutrient broth of aliquots from a mélange of the contents of tubes of lysed animals from the radiation experiments, yielded a psychrophilic Pseudomonas and some Klebsiella. These were most sensitive to the kanamycin, being controlled at 1 μg/ml, so that kanamycin was the antibiotic of choice for the control of commensal organisms.

The next step was to test the sensitivity of planarians to various concentrations of kanamycin. Since concentrations of 10 μg/ml and 100 μg/ml were found to have no effect on D. lugubris B or D. etrusca (exposed for 67 days), 10 μg/ml was used in experiments to test the effect of control of commensal organisms on planarian postirradiation survival.

iii. Kanamycin-induced Bacteriostasis and Asepsis

As was found for the previously tried antibiotics, the parameters of the probit survival curve were not changed significantly, but the survival time was increased by 17% and 81% (D. etrusca and D. lugubris B, respectively) (q.v. table V-4). These increases in survival time were found to be highly significant ($P < 0.001$).

Thus planarian postirradiation death is not, to any

significant degree, a result of infection rather than loss of reproductively competent stem cells (neoblasts).

3 Effect of Antibiotics on the Effect of Postirradiation Feeding

The postirradiation presence of antibiotics did not significantly influence the effects of postirradiation feeding. Both the parameters of the probit survival curve and the time of death were unchanged. Thus the effects of postirradiation feeding were not a result of an increased susceptibility to infection by commensal organisms at a time when the planarian's ability to deal with such organisms may have been diminished.

4 Effect of Postirradiation Decapitation

As the processes of regeneration are known to stimulate the neoblasts into mitotic activity (q.v. Ch. I), and as some further experiments required the cutting of the animals concerned, it was necessary to ascertain the magnitude of the effect of postirradiation decapitation on survival. (The effects of radiation on regeneration are described in Ch. XII.)

The survival curve parameters and the survival times of postirradiation decapitates and their controls are presented in table V-7. Except where otherwise specified, all animals were decapitated 24 $\pm$ 3h postirradiation and were fasted for the duration of the experiment. No significant changes were found in survival curve parameters, nor in survival times, for any of the species examined. The one marginal exception

to this was the fed Polycelis tenuis-hepta as compared with the pooled fed control. The decapitated animals were slightly protected ($P \approx 0.05$). In all cases the experimental animals, prior to decapitation, were of the same size as the controls.

Thus we can conclude that 24h postirradiation decapitation (24h and 48h for Polycelis tenuis-hepta) has little or no significant effect on planarian survival.

5 Effect of Preirradiation Decapitation

Animals irradiated at various times after decapitation might be expected to differ in their survival responses because neoblast mitotic activity and numbers are increased in response to the wound (q.v. Ch. I). The results of 6h and 12h preirradiation decapitation in D. etrusca, and 24h and 48h preirradiation decapitation in D. lugubris B are given in table V-8. Where the relevant controls had larger than usual uncertainties in the estimation of their survival parameters, the survival parameters of the population standards (combined controls from all control experiments relevant to that species and biotype) are given for comparison.

Preirradiation decapitates in all cases produced slight increases in the LD_{50} estimates, but none of the survival curve parameter changes proved to be significant. D, lugubris B times of death also did not change significantly. But significant and highly significant decreases in the respective survival times of 6h ($P < 0.02$) and 12h ($P < 0.001$)

D. etrusca preirradiation decapitates were found.

6 Effect of Age and Sexual State

The physiological age of a planarian is closely related to its body size, which is also a determinant of sexual maturity (q.v. Ch. 1). This suggests that metabolic differences may exist between sexually mature (adult) and sexually immature (young) planarians, which may have an effect on postirradiation survival.

The survival curve parameters and the survival times of young (5-7 mm in length, genital pore absent) as compared with adult (8-12 mm in length, genital pore present) specimens yields no significant differences ($P \gtrsim 0.05$). The data, however, do suggest slightly lower estimates of LD_{50} and K for the young animals (q.v. table V-9). Although no significant differences in survival were found, all experiments were carefully controlled for size of animal; as a routine, only adults were used. The method used to measure the length of a planarian is described in Ch. VI part 1.

7 Determinants of Postirradiation Survival Time

As we noted above, the immediate data to be obtained from a dose-survival experiment are the time of mortality and the parameters of the dose-survival curve. In this section we shall examine the relationship between time of mortality and radiation dose.

a. D. etrusca

Let us consider the results of five standard irradiation experiments, differing only in length of preirradiation starvation (9-20 days) - a factor found not to have any significant influence on the survival curve parameters. We found by both the Corner Test for Association (Olmsted and Tukey, 1947) and analysis of variance that there is no significant regression of time of mortality on dose. The Corner Test yielded a quadrant sum of 2.7 (sums of 9.0 and 11.0 being the lower limits for association at the 10% and 5% confidence levels respectively), which is in good agreement with an $F_{(1, 295)}$ ratio of 36.35 $\angle F(1, 200, 0.05) = F(1, 500, 0.05) = 254 \rangle$. The mean time of death was 34.6 days (297 animals) which is close to the median value of 34 days.

If we now consider the results of three experiments which differed from the above in that an antibiotic (kanamycin, 10 μg/ml) was present in the postirradiation milieu, we find a significant association between dose and time of death - by the Corner Test, P = 0.005, and by analysis of variance $0.01 < P < 0.05$. The regression equation, obtained by the method of Least Squares, is found to be:

$$Y = -6.1 X + 50.6 \quad (n = 98 \text{ animals})$$

time of death in days

X-ray dose in krad

Thus if mean time of death were to be taken as a measure of postirradiation rate of decay of functional vital systems, then the mean time of death of 34.6 days for animals not given kanamycin in the postirradiation milieu is equivalent to that caused by an X-ray dose of 2.6 krad in kanamycin-treated animals. Similarly, the time of death for postirradiation fed animals is equivalent to 4.2 krad in kanamycin-treated animals - however, postirradiation feeding also affected the % survivors.

b. D. lugubris B

If we consider the results of seven standard irradiation experiments in which 265 animals died, in the light of the Corner Test, a quadrant sum of 0.25 is obtained indicating no significant correlation between dose and time of death. The mean time of death was 26.5 $\pm$ 0.4 days postirradiation. Similarly, when kanamycin was added to the postirradiation milieu (10 μg/ml), no significant correlation between dose and time of death was found. However, the mean time of death was increased to 47.1 $\pm$ 2.7 days postirradiation; a highly significant increase ($P < 0.001$).

No significant correlation between time of death and X-ray dose was found for any of the factors investigated in sections C 1 - C 6 of this chapter. The same was true for D. lugubris A and Polycelis tenuis-hepta.

D Results for Standard Populations

On the basis of the results of the experiments described in section C of this chapter, the following conditions were chosen as those where planarian postirradiation death would be most likely to have depletion of reproductively intact neoblasts as the sole determinant.

Animals, 8-12 mm long, were kept in tap water at 20°C and subjected to continuous starvation (until well after the endpoint time) starting at least one week prior to irradiation.[1]

For each of the five species investigated, the results of experiments performed under these conditions were combined to yield the parameters of the standard population response to 300 kVp X-rays. These "population standards" are presented in table V-10.

E Results of X-ray Dose-fractionation Experiments

In order to examine the planarian's capacity to repair radiation-induced sublethal damage, dose-fractionation experiments were performed.

An initial dose (conditioning dose, D_c) of either 800 or 1000 rad was followed at intervals of 4, 6, 8, 12, 24, or 48h, by one of a series of doses sufficient to determine

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1. For the fissiparous species (i.e. D. etrusca, D. tigrina, and Polycelis felina) control of animal size was not practicable, but animals of a mean length of 7 mm were used.

planarian survival curves. A graph of the LD_{50} as a percentage of the single acute dose LD_{50} (control) (figure V-7) shows that even a four hour fractionation interval was long enough for some repair to take place ($P < 0.01$). Although there was no evidence of a significant increase in repair by lengthening the interval between doses from 4h to 24h, the slightly lower LD_{50} for the 6h interval group is suggestive of a heavily damped Elkind-type (Elkind and Sutton, 1959) recovery curve. However, the LD_{50} of the 48h interval group was significantly increased above that of the 24h group ($0.05 > P > 0.01$) and highly significantly above control LD_{50} ($P < 0.001$).

Dose-fractionation was also found to change the slope of the survival curve. However, the pattern was not quite that observed for the LD_{50} (q.v. figure V-8). With the exception of the 8h fractionation interval, the slope of survival curves for all other dose-fractionation intervals was found to be significantly (at 4h, 24h, and 48h) or highly significantly ($P < 0.01$ at 6h and 12h) shallower than that of the control survival curve. The observation that the slope does not return to the control value after 24h or 48h suggests that a relatively long-lived heterogeneity of response to the second dose fraction may be induced by the conditioning dose (q.v. table V-11).

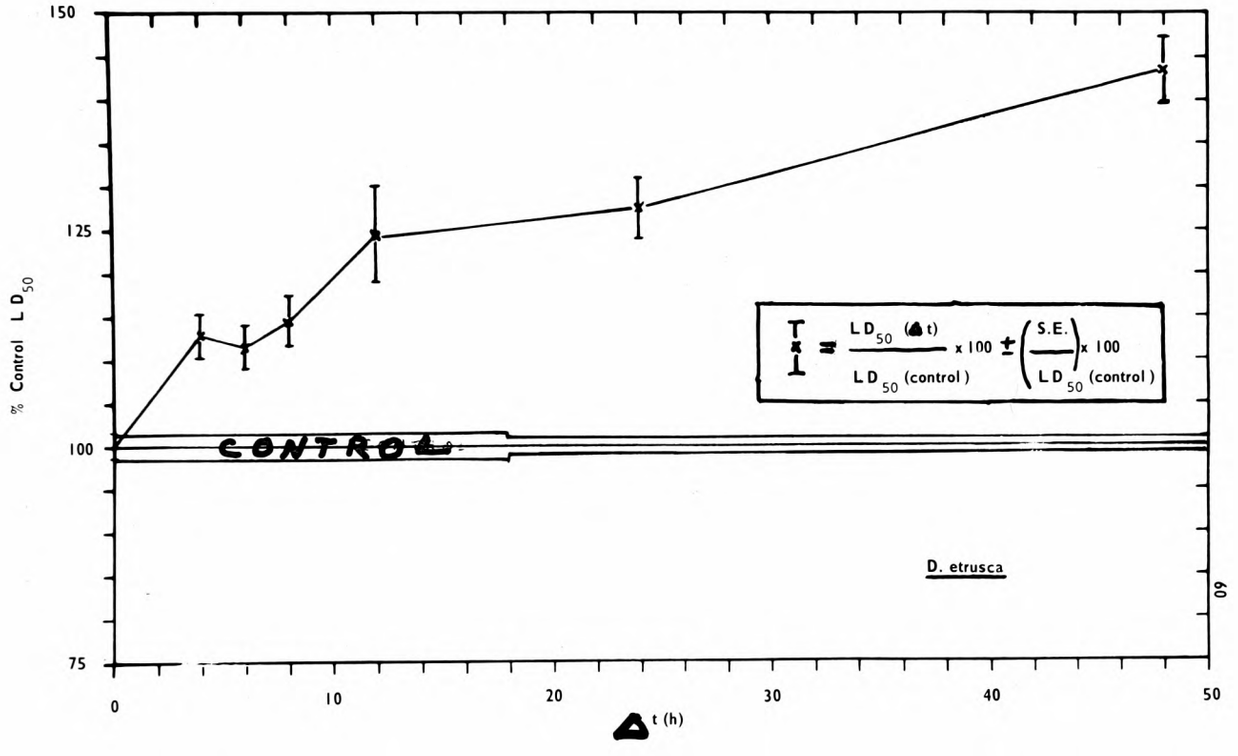

Figure V-7

Effect of dose-fractionation interval on LD_{50}

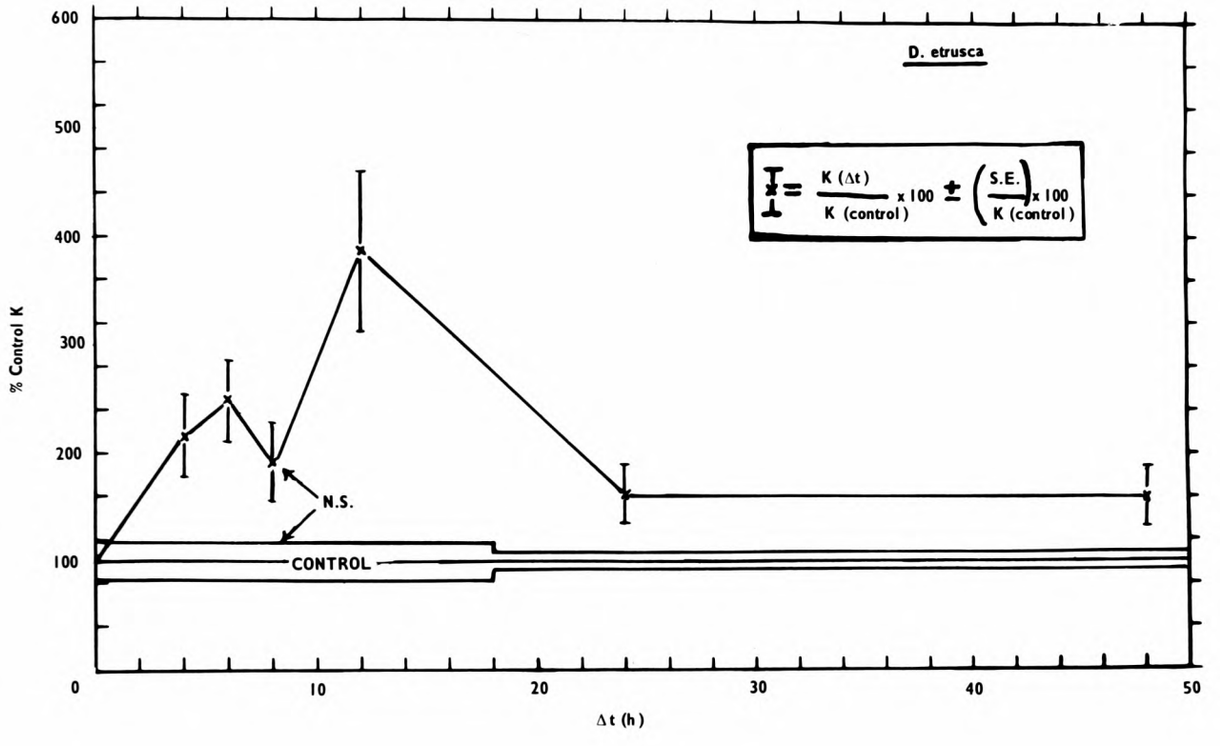

Figure V-8

Effect of dose-fractionation interval on inverse slope

(N.S. = difference not significant)

A graph of the difference between the LD_{50} for each fraction interval and control LD_{50} reveals the pattern of repair of the sublethal damage caused by the conditioning dose, or its converse, the pattern of decay of residual injury (i.e. $1 - (LD_{50} / D_c)$). Such graphs of damage repair and injury decay are shown in figures V-9 and V-10. The data suggest a possible exponential decay of residual injury, but further experiments using longer fractionation intervals are necessary to confirm this hypothesis.

For all the fractionation intervals studied, survival time remained constant at the control value except for the 12h group, for which there was a slight but significant shortening (q.v. table V-12).

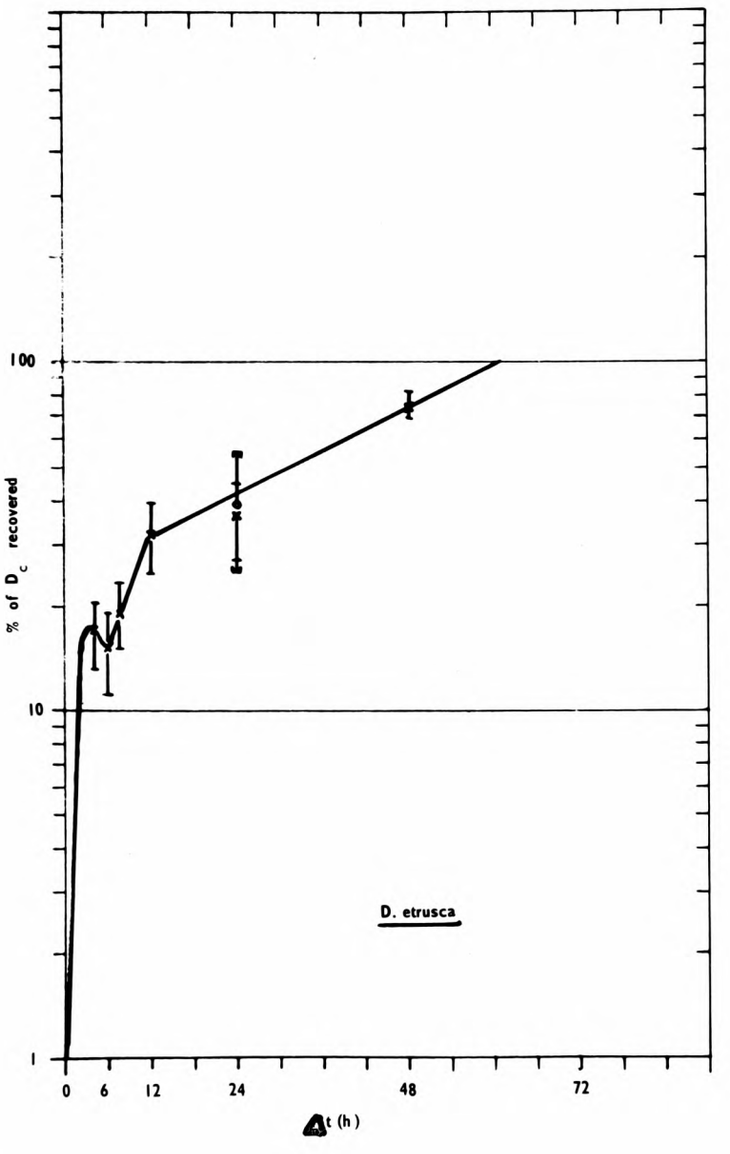

Figure V-9

Effect of dose-fractionation interval on recovery of D_c

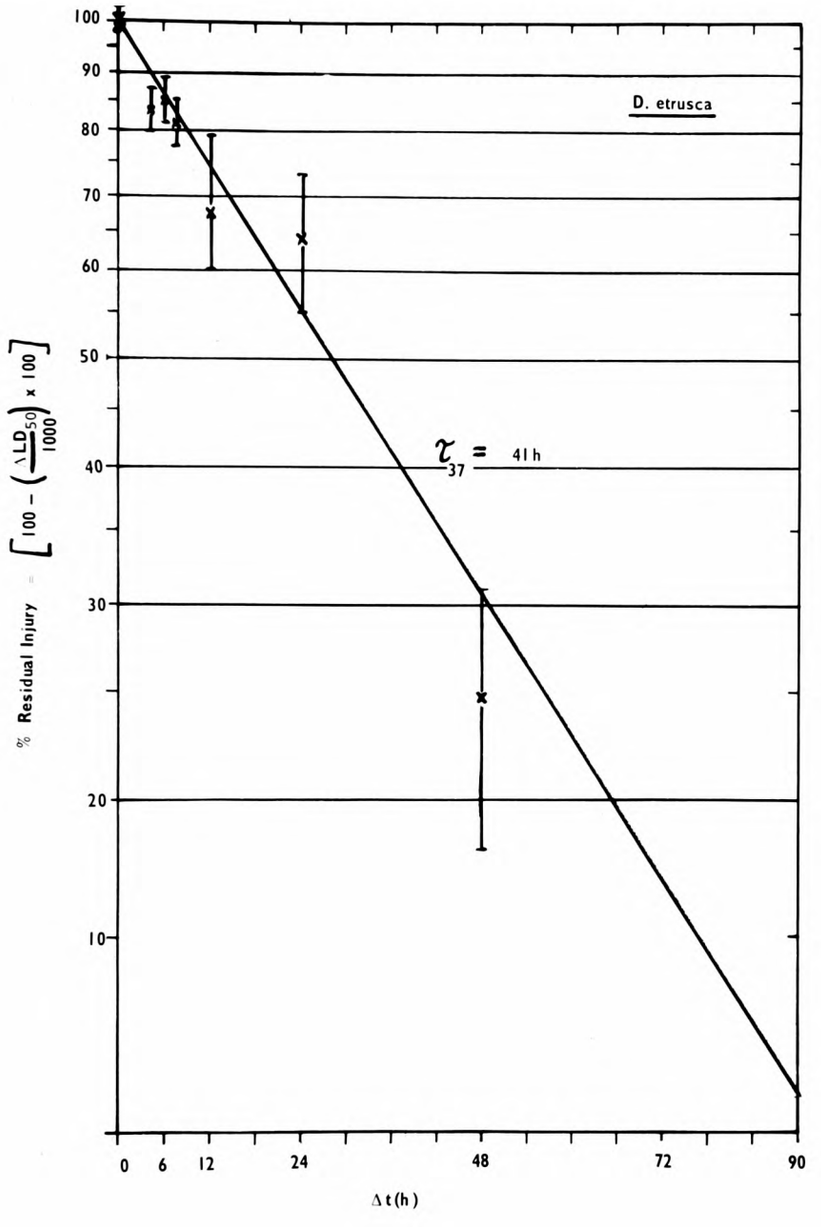

Figure V-10

Effect of dose-fractionation interval on decay of residual injury

TABLE I.

	50% dead.	100% dead.	% survival 60 days.
Fed (2000 RAD)	22 days	32 days	0
Starved (2000 RAD)	38 days	48 days	0
Fed (1000 RAD)	22 days	–	2.5
Starved (1000 RAD)	–	–	72.5

Table V - 1

The Effect of Postirradiation Starvation on the Survival of *D. etrusca*

Table V-2

Effect of Postirradiation Feeding

Species	Condition	No. exps.	ϑ_t	$\bar{t}_{mort}$ ± S.E.	LD_{50} ± S.E.	K ± S.E.	ϑ_s	C ± S.E.
D. etrusca	A. starved (std.1)	4	47	39.1 ± 1.0d	1207 ± 30	269 ± 27	28	1.1 ± 1.2%
	B. fed (1403, 1404)	2	38	25.1 ± 1.2d	*757 ± 854	124 ± 433	11	0 ± 1.6%
D.lugubris	A. starved (std.)	7	52	27.4 ± 0.9d	1677 ± 143	936 ± 206	49	12 ± 5.5%
	B. fed (4565)	2	9	22.5 ± 1.3d	1520 ± 96	565 ± 105	8	8.1 ± 4.3%
Polycelis tenuis-hepta	A. starved (std.)	5	37	58.4 ± 4.9	**706 ± 104	388 ± 94	36	1.9 ± 3.1%
	B. fed (4400, 700)	2	39	30.6 ± 2.0	***718 ± 40	272 ± 49	9	2.5 ± 2.7%

* The large standard errors for the estimates of the probit line parameters for the fed population are due to an insufficiency of data at low doses. That the means, however, serve as useful guides to the magnitude of the effect, is confirmed by the significance of the difference in survival level ($p < 0.001$) at 1000 rads (q.v. table IV-1).

** at 120d postirrad.

*** at 60-120d postirrad.

Table V-3

Survival Parameters and Survival Time Comparisons for Different Durations of Preirradiation Starvation

	days pre-starved	σ_s	LD_{50} ± S.E.	K ± S.E.	$\bar{t}_{mort}$ ± S.E.	σ_t	C ± S.E.
D.etrusca							
XP 1001	4	8	1311 ± 60	262 ± 57	37.6 ± 1.6	4	0 ± 1.3%
702	11	3	961 ± 61	148 ± 85	36.2 ± 7.6	4	2.5 ± 1.8%
(1) 1200	16	4	1272 ± 36	208 ± 36	39.5 ± 0.5	17	0.4 ± 2.1%
1401	20	4	1090 ± 44	219 ± 50	39.8 ± 1.4	19	0 ± 2.2%
XP 92	9	5	1369 ± 30	185 ± 33	36.9 ± 1.0	17	1.2 ± 1.3%
12000	9	2	1289 ± 163	57 ± 241	24.0 ± 0.6	24	0 ± 2.2%
12403	9	2	651 ± 936	1361 ± 1140	31.6 ± 1.5	4	0 ± 3.2%
10701	12	6	1398 ± 29	178 ± 35	41.8 ± 2.9	12	0 ± 2.2%
(2) 93	14	5	1413 ± 29	159 ± 31	32.7 ± 0.6	30	0 ± 1.1%
10801	14	2	1323 ± 28	108 ± 26	30.0 ± 1.8	23	0 ± 2.2%
87	16	5	1366 ± 61	220 ± 62	39.0 ± 0.8	23	0 ± 2.1%
96	16	5	1419 ± 25	118 ± 23	31.6 ± 0.3	28	0 ± 1.1%
99	20	4	1310 ± 32	174 ± 31	35.4 ± 0.6	19	0 ± 1.6%
D.lugubris B							
XP 701	5	8	1201 ± 61	150 ± 53	21.4 ± 1.5	6	0 ± 1.6%
6301	5	6	1737 ± 211	408 ± 222	30.1 ± 2.1	9	22.3 ± 13.2%
7601	5	4	1151 ± 497	2285 ± 905	29.6 ± 0.8	23	15.3 ± 8.5%
3400	6	3	1757 ± 292	972 ± 440	24.1 ± 2.1	8	0 ± 3.3%
4703	20	4	1772 ± 491	1184 ± 1360	30.1 ± 2.9	13	0 ± 8.1%
5212	20	2	1400 ± 366	400 ± 343	23.8 ± 1.2	13	39.8 ± 21.1%
3800	29	4	1855 ± 127	264 ± 163	27.9 ± 1.2	11	26.7 ± 10.5%
D.lugubris A							
XP 7501	5	4	2907 ± 93	356 ± 126	29.2 ± 1.9	15	7.5 ± 2.8%
7701	5	6	2400 ± 204	760 ± 233	37.4 ± 1.9	23	0 ± 3.1%
5112	20	2	2098 ± 307	703 ± 471	21.8 ± 4.1	4	2.2 ± 5.4%
Polycelis tenuis-hepta							
XP 6201	5	5	981 ± 235	564 ± 257	47.2 ± 11.6	5	8.6 ± 12%
4302	20	5	540 ± 256	265 ± 179	63.1 ± 5.3	10	0 ± 3.2%
4903	20	4	726 ± 279	353 ± 206	47.9 ± 9.2	7	0 ± 3.2%
5304	20	6	800 ± 135	151 ± 150	58.4 ± 13.9	4	0 ± 7.1%
5401	20	4	581 ± 433	786 ± 383	70.8 ± 11.2	7	0 ± 4.4%

Table V-4

Effects of Antibiotics on Survival

Species		Treatment	LD_{50} ± S.E.	K ± S.E.	σ_s	$\bar{t}_m$ ± S.E.	σ_t
D.etrusca	(1)	Control	1090 ± 44	219 ± 50	4	39.8 ± 1.4	19
"	(1)	Chloramphenicol	1112 ± 39	203 ± 43	4	45.2 ± 1.0	18
"	(1)	Penicillin + Streptomycin	1191 ± 38	218 ± 42	4	48.2 ± 0.9	12
"	(2)	Control	1359 ± 19	163 ± 18	20	35.1 ± 0.5	72
"	(2)	Kanamycin	1408 ± 30	214 ± 32	20	41.2 ± 0.6	79
"	(1)	Chloramphenicol fed	757 ± 854*	124 ± 433*	11	24.4 ± 1.9	18
"	(1)	Control fed				25.7 ± 1.6	19
D.lugubris B		Control	1677 ± 143	936 ± 206	49	26.2 ± 0.7	97
"		Kanamycin	1971 ± 123	511 ± 140	4	47.1 ± 2.7	19

* Curve fitting errors are abnormally high as a result of the paucity of survivors at the lowest irradiation dose used (1000 rad).

Table V-5

Identification of Organisms

Tube Contents	Gram -ve bacilli	Gram +ve cocci	Pseudomonas aeruginosa	Apsychrophilic Pseudomonas	Streptococcus ∝ haemolyticus	Klebsiella
Tap water	+	+	-	-	-	-
Tap water + food	+	+	-	-	-	-
Tap water + food + D.lugubris B	-	-	+	-	+	-
Tap water + food + Polycelis felina	-	-	+	-	+	-
Tap water + irradiated D.lugubris B	+	+	-	-	-	-
Mélange of irradiated planarian lysates	-	-	-	+	-	+

Table V-6

Antibiotics Tested

Antibiotic**	Concentration	Resistant*
Kanamycin	30 µg/disc	None
"	1 µg/ml	None
Colomycin	200 µg/disc	None
Noxytiolin	10 mg/disc	5
Negram		2, 4
Chloramphenicol	50 µg/disc	4
"	25 µg/ml	1, 3-5
Penicillin and Streptomycin	2500 units each/ml	1, 4, 5
Nitrofurantonin	200 µg/disc	3-5
Penbritin		1, 3-5
Neomycin	10 µg/disc	2-5
Streptomycin	25 µg/disc	1, 3-5
Cloxacillin	5 µg/disc	1, 3-5
Sulphonamide		2-5
Nystatin	50 units/disc	1-5
Penicillin	2500 units/ml	1-5

* Source of Resistant Organisms
1. water only
2. water + food
3. water + food + planarian (lysed)
4. water + irradiated planarian (lysed)
5. mélange of irradiated planarian lysates.

** Oxoid Multodisks 30-9C and U-2.

Table V-7

Survival Parameters and Survival Times for 24h Postirradiation Decapitated Animals and their Controls

	LD_{50}	± S.E.	σ_s	K	± S.E.	$\bar{t}_{mort}$	± S.E.	σ_t	C	± S.E.
D.etrusca										
Control 1401	1090	± 44	4	219	± 50	39.8	± 1.4	19	0	± 2.2%
Decap. 1406	1178	± 56	4	228	± 65	39.8	± 0.5	7	0	± 3.2%
D.lugubris B										
Control 4703	1772	± 491	4	1184	± 1360	30.1	± 2.9	13	0	± 8.1%
Decap. 4704	1764	± 192	4	632	± 289	23.9	± 2.0	14	0	± 5.1%
Control 6301	1737	± 211	6	408	± 222	30.1	± 2.1	9	22.3	± 13.2%
Decap. 6303	1658	± 275	6	698	± 251	25.6	± 1.5	13	28.5	± 17.6%
Control 7601	1151	± 497	4	2285	± 905	29.6	± 0.8	23	15.3	± 8.5%
Decap. 7603	2447	± 281	4	1076	± 436	31.0	± 2.2	16	14.2	± 7.3%
*Pooled Control	1677	± 143	49	936	± 206	29.9	± 1.0	47	12.0	± 5.5%
Pooled Decap.	1940	± 187	20	910	± 256	27.0	± 1.2	45	11.8	± 7.1%
D.lugubris A										
Control 7501	2907	± 93	4	356	± 126	29.2	± 1.9	15	7.5	± 2.8%
Decap. 7503	5250	± 3320	4	2443	± 2410	32.9	± 3.5	6	0	± 3.5%
Control 7701	2400	± 204	6	760	± 233	37.4	± 1.9	23	0	± 3.1%
Decap. 7703	3046	± 369	4	919	± 369	44.3	± 5.6	5	0	± 2.2%
*Pooled Control (std.)	2674	± 177	18	873	± 191	34.2	± 1.5	39	4.1	± 4.2%
Pooled Decap.	3620	± 562	11	1348	± 472	38.2	± 3.5	12	0	± 1.9%

Table V-7 (continued)

	LD_{50}	± S.E.	σ_s	K	± S.E.	$\bar{t}_{mort}$	± S.E.	σ_t	C	± S.E.
Polycelis tenuis-hepta										
Control 6201	981	± 235	5	564	± 257	47.2	± 11.6	5	8.6	± 12%
Decap. 6203	1249	± 130	3	597	± 153	44.8	± 6.7	8	0	± 3.2%
Pooled Control (std.)	706	± 104	36	388	± 94	58.4	± 4.9	37	1.9	± 3.1%
**Control 7000	786	± 52	3	318	± 63	38.0	± 3.2	17	0	± 2.2%
**Decap. 7200	950	± 82	3	363	± 101	46.1	± 3.0	17	0	± 2.3%
**Pooled Control (std.)	718	± 40	9	272	± 49	30.6	± 2.0	39	2.5	± 2.7%
**48h Decap. 7300	931	± 62	3	295	± 77	32.4	± 2.0	17	0	± 1.8%

* The survival curve parameters are estimated from all control experiments, but the $\bar{t}_{mort}$ is estimated from only those control populations shown in this table.

** Experiments 7000, 7200, 7300, and the pooled control were postirradiation fed experiments.

Table V-8

Survival Parameters and Survival Times for Pre-irradiation Decapitated Animals and their Controls

	LD_{50} ± S.E.	σ_s	K ± S.E.	E_{mort} ± S.E.	σ_t	C ± S.E.
D. etrusca						
– 12h	1416 ± 97	2	258 ± 90	25.8 ± 3.2	16	0 ± 3.5%
– 6h	1526 ± 176	2	547 ± 195	25.2 ± 2.1	33	0 ± 4.1%
Control	641 ± 936	2	1361 ± 1140	31.6 ± 1.5	4	0 ± 3.2%
Pooled Control	1380 ± 14	36	168 ± 14	34.8 ± 0.4	121	0.1 ± 0.5%
D. lugubris B						
- 48h	2067 ± 141	4	458 ± 187	29.0 ± 1.1	13	0 ± 3.8%
- 12h	1904 ± 95	4	448 ± 127	27.8 ± 1.6	14	0 ± 3.2%
Control	1772 ± 491	4	1184 ± 1360	30.1 ± 2.9	13	0 ± 8.1%
Pooled Control	1677 ± 143	49	936 ± 206	29.9 ± 1.0	27	12.0 ± 5.5%

Table V-9

Survival Parameters and Survival Times for Sexually Immature young vs. Sexually Mature Adults

	LD_{50} ± S.E.	ν_s	K ± S.E.	$\bar{t}_{mort}$ ± S.E.	ν_t	C ± S.E.
Polycelis tenuis-hepta						
fed young 7400	629 ± 35	3	131 ± 36	36.9 ± 2.8	27	9.3 ± 4.4%
fed adult 7000	786 ± 52	3	318 ± 63	38.0 ± 3.2	17	0.1 ± 2.2%
Pooled fed adults (4400) (7000)	718 ± 40	9	272 ± 49	30.6 ± 2.0	39	2.5 ± 2.7%
D. lugubris B						
young 5219	1276 ± 112	2	466 ± 130	25.7 ± 1.6	12	0 ± 3.2%
adults 5212	1400 ± 366	2	400 ± 343	23.8 ± 1.2	13	39.8 ± 21.1%
young 6700	1701 ± 117	4	387 ± 127	29.7 ± 2.5	18	0 ± 4.2%
adults 6800	1572 ± 2100	4	1005 ± 2890	30.0 ± 2.7	19	23.1 ± 29.9%
Pooled young (5219) (6700)	1599 ± 99	9	456 ± 116	28.1 ± 1.6	31	0 ± 3.2%
Pooled adults (Std.)	1677 ± 143	49	936 ± 206	27.4 ± 1.7	33	12.0 ± 5.5%

Table V-10

Population Standards - Survival Curve Parameters

Species	Biotype	LD_{50} ± S.E.	K ± S.E.	σ_s	Heterog.**	C ± S.E.
D. lugubris	A	2674 ± 177	873 ± 191	18	+++	4.1 ± 4.2%
"	B	1677 ± 143	936 ± 206	49	+++	12.0 ± 5.5%
"	D	2141 ± 308	1180 ± 335	5	-	8.8 ± 9.1%
D. etrusca	1.	1207 ± 30	269 ± 27	28	-	1.1 ± 1.2%
	2.	1380 ± 14	168 ± 14	36	-	0.1 ± 0.6%
D. tigrina		977 ± 55	148 ± 54	20	+++	3.9 ± 2.8%
Polycelis tenuis-hepta (fed)		718 ± 40	272 ± 49	9	-	2.4 ± 2.7%
Polycelis tenuis-hepta *(starved)		706 ± 104	388 ± 94	36	++	1.9 ± 3.1%
Polycelis felina		1650 ± 126	508 ± 137	36	+++	8.4 ± 7.4%

* Endpoint time = Day 100 postirradiation

** Non-systematic heterogeneity. - = $P > 0.05$, + = $0.05 > P > 0.01$, ++ = $0.01 > P > 0.001$, +++ = $P < 0.001$. All standard errors are corrected for this heterogeneity.

Table V-11

Results of X-ray Dose-fractionation

Δt	LD_{50} ± S.E.	K ± S.E.	ν_s	C ± S.E.	$\bar{t}_m$ ± S.E.	ν_t
Control (4-12h)	1314 ± 19	95 ± 17	7	0 ± 1.6%	27.0 ± 1.0	48
4h	1482 ± 33	206 ± 33	7	0 ± 1.6%	26.7 ± 1.3	29
6h	1464 ± 34	237 ± 36	7	0 ± 1.8%	25.8 ± 1.2	29
8h	1504 ± 38	182 ± 35	7	0 ± 2.0%	27.6 ± 2.0	20
12h	1635 ± 72	368 ± 70	7	0.2 ± 2.4%	24.3 ± 0.4	41
24h	1759 ± 50	273 ± 45	9	0 ± 1.0%	35.3 ± 0.6	25
48h	1982 ± 51	269 ± 48	9	0.5 ± 1.2%	36.1 ± 3.2	14
*Control (24-48h)	1380 ± 14	168 ± 14	36	0.1 ± 0.5%	37.8 ± 2.0	21

* The survival curve parameters of the controls were in excellent agreement with those of the relevant population standard, so the standard with its smaller variance was substituted. $\bar{t}_m$, however, is calculated from the controls of these experiments.

Table V-12

Repair of Residual Injury in D. etrusca

Δt	D_c (rad)	$\frac{\Delta LD_{50}}{-D_c} \times 100\%$	R.I.(%)	± S.E.(%)
Control	0	0	100	1.4
4h	1000	16.8	83.2	3.7
6h	1000	15.0	85.0	3.9
8h	1000	19.0	81.0	4.2
12h	1000	32.1	67.9	7.4
24h	1000	36.0	64.0	8.8
24h	800	39.5	60.5	14.9
48h	800	75.3	24.7	6.6

Chapter VI

The Model

Chapter VI

The Model

The preceding chapter has established the parameters of planarian mortality at the level of the organism under conditions where the only likely cause of death can be ascribed to a cellular mechanism, i.e. loss of neoblast reproductive integrity. In order to deduce the parameters of the cellular response from the whole animal survival data, the following model was constructed.

Let us assume that the neoblast radiation response (i.e. survival of reproductive integrity) can be described by a logarithmic survival curve of the form

$q = E \exp(-D/D_o)$ where E is the extrapolation number, and let a dosage x be defined as

$x = D/D_o$

where, D - is the radiation dose in rad, and

D_o - is the (as yet unknown) 37% dose-slope of this assumed survival curve. We also define

α - as the probability that a single surviving neoblast repopulates the animal, and

N - as the initial number of neoblasts in the animal ($\approx 3 \times 10^4$, Brøndsted, 1961; but see Chapter VII).

Then the mortality probability for the animal is

$$P_m = (1 - \alpha q)^N$$

$$\approx \exp(-N\alpha q) \qquad \text{if N is large}$$

$$\approx \exp(-N\alpha E \exp(-x))$$

or

$$P_m = \exp(-\exp(-y)) \qquad \text{where } x = x_o + y,\ n = N\alpha E$$

and $n \exp(-x_o) = 1$, or

$x_o = \ln n$.

When we plot P_m vs. y (figure VI-1) to obtain a normalized ($x = D/D_o$) mortality curve, and fit the best straight line to it, we find for the normalized curve

$$x = x_o + y$$

$$LD_{50} = x_o + 0.65 \pm 1.2$$

in units of dosage — value of y at 50% intercept — K for that best straight line $= \frac{y(2.5\%) - y(50\%)}{2}$

For the experimental curve we have measured

$$LD_{50} = 1380 \pm 168 \text{ (e.g. for } \underline{D. etrusca})$$

in rad — K for experimental probit regression line.

Since (experimental values) = (D_o)(normalized values),

$$D_o = \frac{168}{1.2} = 140 \text{ rad } (\pm 11.7 \text{ rad S.E. of population response}).$$

Thus the D_o is equal to the inverse slope of the appropriate animal survival curve, divided by 1.2. The D_o's calculated for each species and biotype investigated are presented in table VI-1.

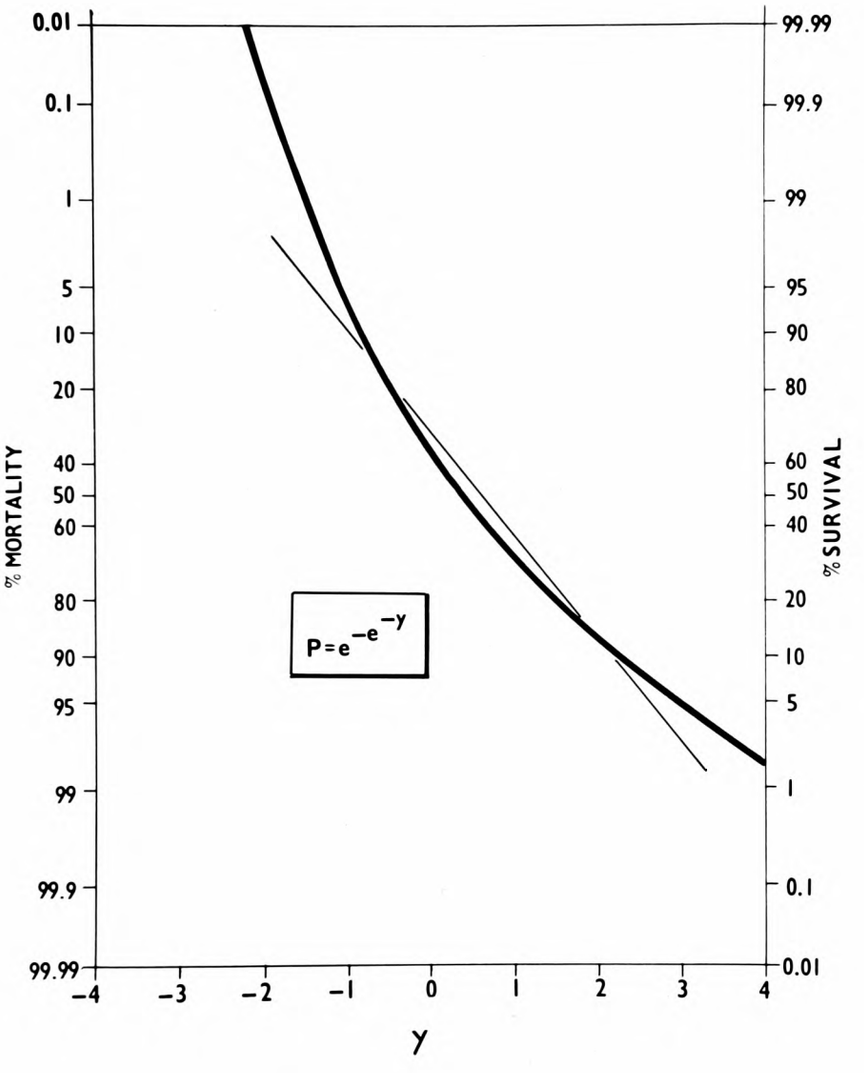

Figure VI-1

Probit Graph of P_m vs y (heavy curve) with straight line of best fit (thin line)

Using the values derived from the inverse slopes of the whole animal survival curves, we can proceed to convert the experimentally measured LD_{50}'s in units of rad into normalized LD_{50}'s in units of dosage:

$$\frac{LD_{50}}{D_o} = x_o + 0.65 ,$$

$$\frac{LD_{50}}{D_o} - 0.65 = x_o$$

$$= \ln n$$

$$= \ln (N\alpha E)$$

so that:

$$N\alpha E = \exp \left[(LD_{50}/D_o) - 0.65 \right] .$$

For D. etrusca, for example,

$$N\alpha E = \exp \left[(1380/140) - 0.65 \right]$$

$$= \exp (9.207)$$

$$\approx 10,000 .$$

The values of $N\alpha E$ for each of the species and biotypes studied are given in table VI-1. The standard errors (S.E.) of table VI-1 are based on the standard errors of the animal LD_{50} and K estimates, appropriately transformed. The error of the transformation was estimated to be negligible with respect to the errors of the animal survival curve.

This model predicts values of D_o, and of the product $N\alpha E$. In order to define the extrapolation number, E, of the neoblast survival curve, N and α must first be determined.

Chapters VII and VIII are concerned with experiments to measure these two parameters, N and α, respectively. As we are dealing with survival or mortality probabilities and the dose to yield 50% survivors is the dose of greatest accuracy of determination,, it is the value of α which corresponds to that survival probability, the α_{50} which shall concern us.

Table VI-1

Values of D_o and $N\alpha E$

Species	Biotype	$D_o \pm$ S.E.	$N\alpha E \pm$ S.E.	$\left(\frac{S.E.(N\alpha E)}{N\alpha E}\%\right)$
D. lugubris	A	727 ± 159	20.66 ± 15.03	73
"	B	780 ± 172	4.48 ± 2.39	53
"	D	983 ± 279	4.61 ± 3.74	81
D. etrusca	1.	224 ± 23	114.20 ± 66.2	58
"	2.	140 ± 12	9970 ± 8060.	81
D. tigrina		123 ± 45	1470 ± 1715.	117
Polycelis tenuis-hepta (fed)		226 ± 40	9.51 ± 16.6	175
Polycelis tenuis-hepta *(starved)		324 ± 79	4.62 ± 3.68	80
Polycelis felina		423 ± 114	25.79 ± 30.6	119

* Endpoint time = D_{100} postirradiation

Chapter VII - Part 1

Histological Methods and Results

Chapter VII- Part 1

Histological Methods and Results

The histochemistry, distribution, and factors which influence the differentiation of the planarian neoblast have been studied in several species, as noted in Chapter I. Brøndsted and Brøndsted (1961) have published the only reported estimate of the total number of neoblasts in a planarian. This chapter presents information about the number of neoblasts in diploid, triploid, and tetraploid D. lugubris . The relation of this number to the size of the animal, and the distribution of neoblasts with respect to the animal's long axis are also described.

METHODS

Material

The animals used for this study were the diploid (A), triploid (B) and tetraploid (D) biotypes (Benazzi, 1957) of a naturally occurring polyploid series of Dugesia lugubris (O. Schmidt). Only animals which had hatched from cocoons laid in the laboratory were used, so that the size of each animal could be taken as a measureof its age (c.f. Reynoldson, 1961) i.e. the cell counts for small animals were

not influenced by possible variations due to shrinkage of starved animals.

Measurement of size

The length of each planarian was measured by placing the living animal in a very shallow depth of water in a petri dish directly on mm graph paper. At a magnification of x10, the length of the animal was determined to the nearest 0.5 mm while it was swimming in a relaxed and extended condition. This method yielded results which were in good agreement with those obtained by measuring photographs of planarians swimming on a mm grid.

Histological Methods

Animals of various sizes were starved for six days before killing, in the relaxed and extended position, either with a drop of 2% HNO_3 (Hyman, 1924), or by placing the animal in a small drop of water between a glass slide and a No. 0 coverglass prior to fixation in Susa or Formol-Zenker. Specimens were serially sectioned (frontally) at 10μ on a rotary microtome. The material reported on was stained with 25% Azure B in pH 4.0 McIlvainie's buffer and differentiated in tert.-butanol at 40°C for 18-22 hrs. (18 hrs. for Susa-fixed material, 22 hrs. for Formol-Zenker).

Azure B is specific for DNA and RNA and does not fade (Flax and Himes, 1952). RNA specificity was tested by RNase preincubation, resulting in a total absence of blue or purple

staining material (RNA). The RNA specificity of a stain is an important aid in the recognition of neoblasts (Benazzi-Lentati, 1943: Pedersen, 1959b). The neoblast is easily recognized due to its peculiar morphology, i.e. a nucleus of ~7 μ diameter surrounded by a narrow (~1 μ) band of RNA-rich cytoplasm.

Cell Counts

Neoblasts, or parts of neoblasts, were recognized by their staining properties and all of them were counted at x400. Twenty per cent of the sections (two every 100 μ along the cephalo-caudal axis of each planarian) were counted for twelve triploids and seven diploids.

In order to check the reliability of the counts, slides were chosen at random for recounting. For small animals (2 mm live length) the median variation for one section, and the variation in the total number of neoblasts summed over the recounted sections were both less than 5%, while for large animals (10 mm live length), the variations were 12-13% and $< 0.5\%$ respectively.

The results which follow are based on 20% sample counts of nine tetraploids, twelve triploids and seven diploids. Serial sections were made of twelve tetraploids, twenty-eight triploids and eleven diploids but since there was good agreement between the results obtained from animals of the same size (live length), only two or three specimens of a given ploidy were counted for each size.

Measurement of Nuclear Diameter

Measurements of nuclear diameters (the mean of 2 measurements, perpendicular to each other, per cell) were made at x844 magnification for diploid and triploid animals, with an ocular micrometer (Leitz) to the nearest 0.3μ.

Measurement of Planarian Volume

The area of every twentieth section was measured at a magnification of x187.5 with a Leitz eye-piece micrometer. The area of the parenchyma plus gut was obtained by subtracting the area enclosed by the pharynx sheath from that of the entire section. These areas were graphed against position on the anterior-posterior axis of the animal and volumes were obtained by measuring the area under the curves.

RESULTS

A Reliability of Data

Estimates of the number of neoblasts in a given interval along the anterior-posterior axis of a planarian, based on the 20% sample taken, were not significantly different ($P > 0.05$) from those obtained by counting every section in that interval. The 20% sample yields estimates of the number of neoblasts per slide within 10% of the actual count; and summed over several slides, the difference drops to ~1%. This is of the order of the variations found in recounts for large animals (10-12 mm), i.e. 12-13% for single sections and <0.05% for sums over several slides.

B Total number of neoblasts in a planarian as a function of its length

The relationship between the length of a planarian and its total number of neoblasts is shown in figure VII-1.[1] The data for diploids, triploids and tetraploids were handled separately and the best line for each set of data was fitted by the method of least squares. Graphing the data on Cartesian and on semi-logarithmic coordinate scales showed strong nonlinearity, whereas if both variables were transformed logarithmically, linearity was evident. The equations for the three regression lines are:

$\log N = 1.90 \log L + 3.53$ for the diploids,

$\log N = 1.93 \log L + 3.41$ for the triploids,

and $\log N = 1.71 \log L + 3.74$ for the tetraploids

where N is the total number of neoblasts per animal[1] and L is the length, in mm, of the living animal.

Analysis of variance (c.f. Bennett and Franklin, 1954) for linearity of regression yielded F values of 369 for the diploids ($F_{1,5,\ 0.001} = 47.2$), 534 for the triploids ($F_{1,10,\ 0.001} = 21.0$), and 798 for the tetraploids ($F_{1,7,\ 0.001} = 29.3$), indicating that the probability of obtaining such a degree of linearity by chance is much less than 0.001.

---

1. In the Results sections A, B and C of this chapter, the number of neoblasts in any one section, or in any part of a serially-sectioned planarian comprising more than one section, is taken to be the number of neoblasts counted in that section or the sum of the number of neoblasts in each of the relevant sections. Corrections for overcounting due to some cells appearing in more than one section will be considered under Results section D, and in Appendix III.

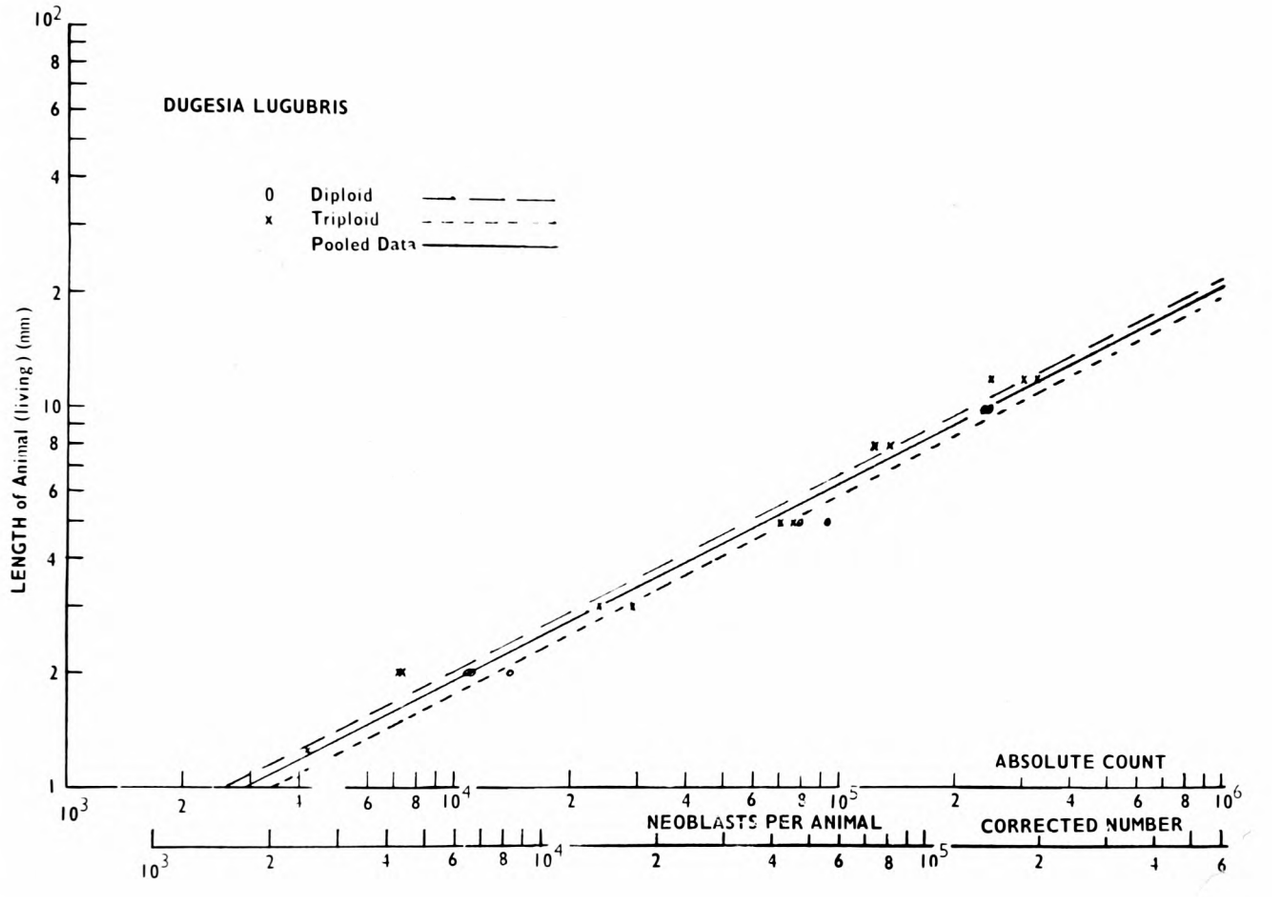

Figure VII-1

Relationship between planarian length and number of neoblasts (D. lugubris)

Data points and regression lines for number of neoblasts per animal on length of animal when alive. The lower scale is obtained by applying Abercrombie's correction to the crude count (upper scale). Each point represents one animal (12 triploids and 7 diploids).

The significance of the differences in slope and intercept of the regression lines were examined by means of the "t-test", yielding a "t" value for the diploid-triploid slope difference of $t_{15} = 0.2173$ ($P > 0.7$) and a "t" value for the diploid-triploid intercept difference of $t_{15} = 1.2373$ ($P > 0.1$). Similarly, no significant differences were found between diploid-tetraploid and triploid-tetraploid slope and intercept comparisons. Thus the number of neoblasts in a diploid planarian of a given size is not significantly different from that in a triploid or a tetraploid of the same size.

Combining the results for diploid and triploid animals yields a regression equation of:

$$\log N = 1.90 \log L + 3.47,$$ [1]

and inclusion of the results for the tetraploids yields a regression equation of:

$$\log N = 1.95 \log L + 3.46$$

C Antero-posterior distribution of neoblasts

In order to be able to compare the antero-posterior distribution of neoblasts in animals of various sizes, over the wide range of neoblast numbers and planarian lengths, both variables had to be normalized. The independent variable, length, was normalized by taking class intervals of 10% of the total (fixed) length. The dependent variable was normal-

1. The value of N, for diploid and triploid animals, used in the chapters which follow are derived from this equation as the tetraploid data were not available until shortly before this thesis was typed.

ized by setting the area under the distribution curve for each animal equal to one, i.e. for each class interval the proportion of the animal's total neoblast count appearing in that interval was recorded. The agreement of distribution curves between animals of the same size and ploidy was good; as is shown, e.g. by the data for 12.0 mm triploids, in table VII-1. The next row gives for each interval, the mean of the proportions of neoblasts found in that interval; and the following row, the standard deviations of those means. The two maxima, one in the third and fourth intervals and the other in the eighth interval, with an intercalary relative minimum in the sixth and seventh intervals, are characteristic for the triploid Dugesia lugubris.

In figure VII-2, we see that the normalized distribution curves for 2.0, 3.0, 5.0, 8.0 and 12.0 mm long triploids are nearly superimposable, the relative minimum corresponding to the position occupied by the pharynx, occurring in the sixth and seventh intervals for all sizes. In contradistinction to the near superimposability of the triploid distribution curves for all sizes, the diploids show a distinct progression in the position of the pharynx as the animals increase in size (figure VII-3). The pharynx moves from the seventh interval in the 2.0 mm animal (freshly hatched), to the sixth interval in the 5.0 mm animal (immature), to the fifth interval in the 10.0 mm animal (sexually mature adult). The

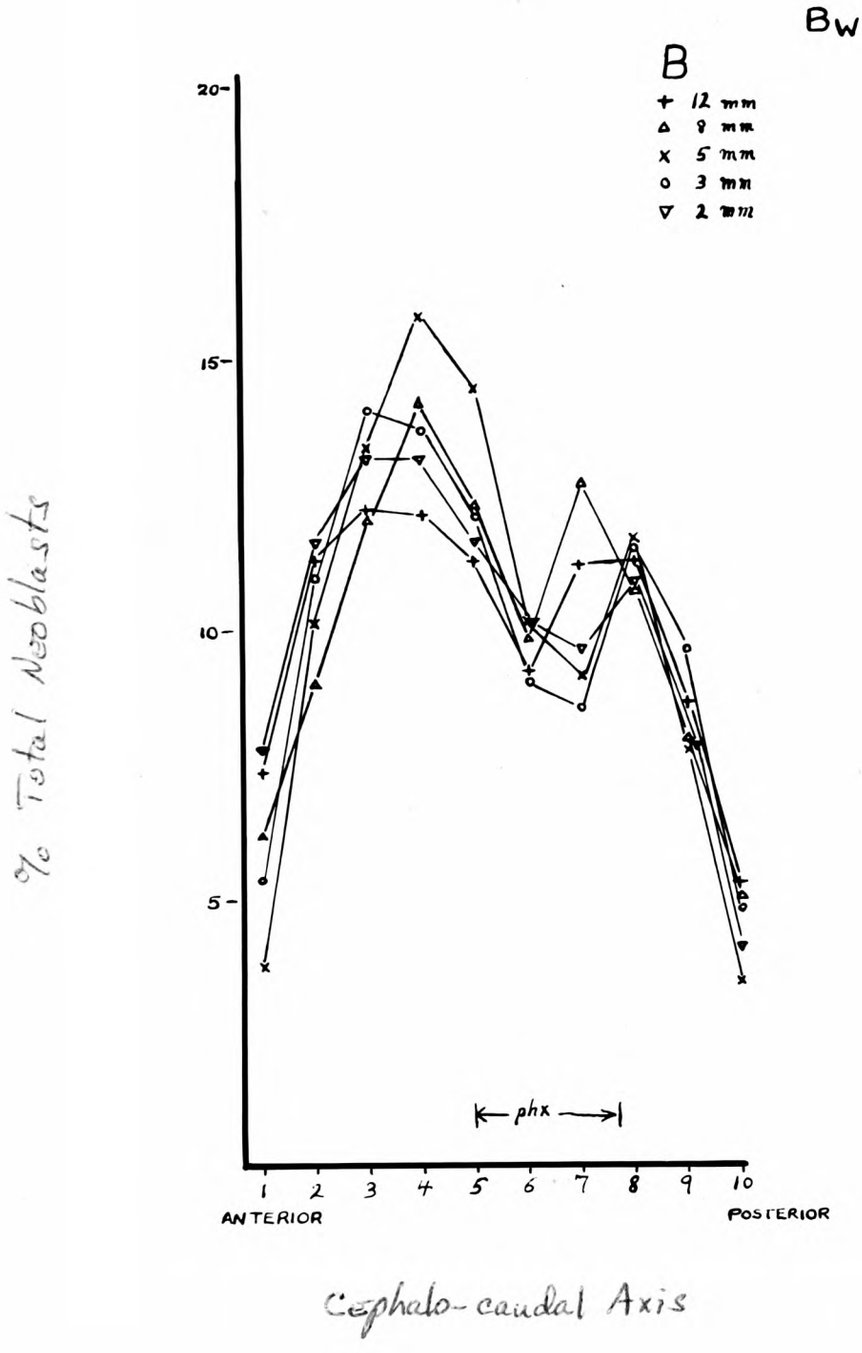

Figure VII-2

Normalized neoblasts distribution curves for D. lugubris B

The percentage of a (triploid) planarian's neoblasts to be found in each 10% interval in length along the cephalo-caudal axis is shown. Each point represents the mean for two or three animals. The arrows show the anterior and posterior limits of the pharynx (phx).

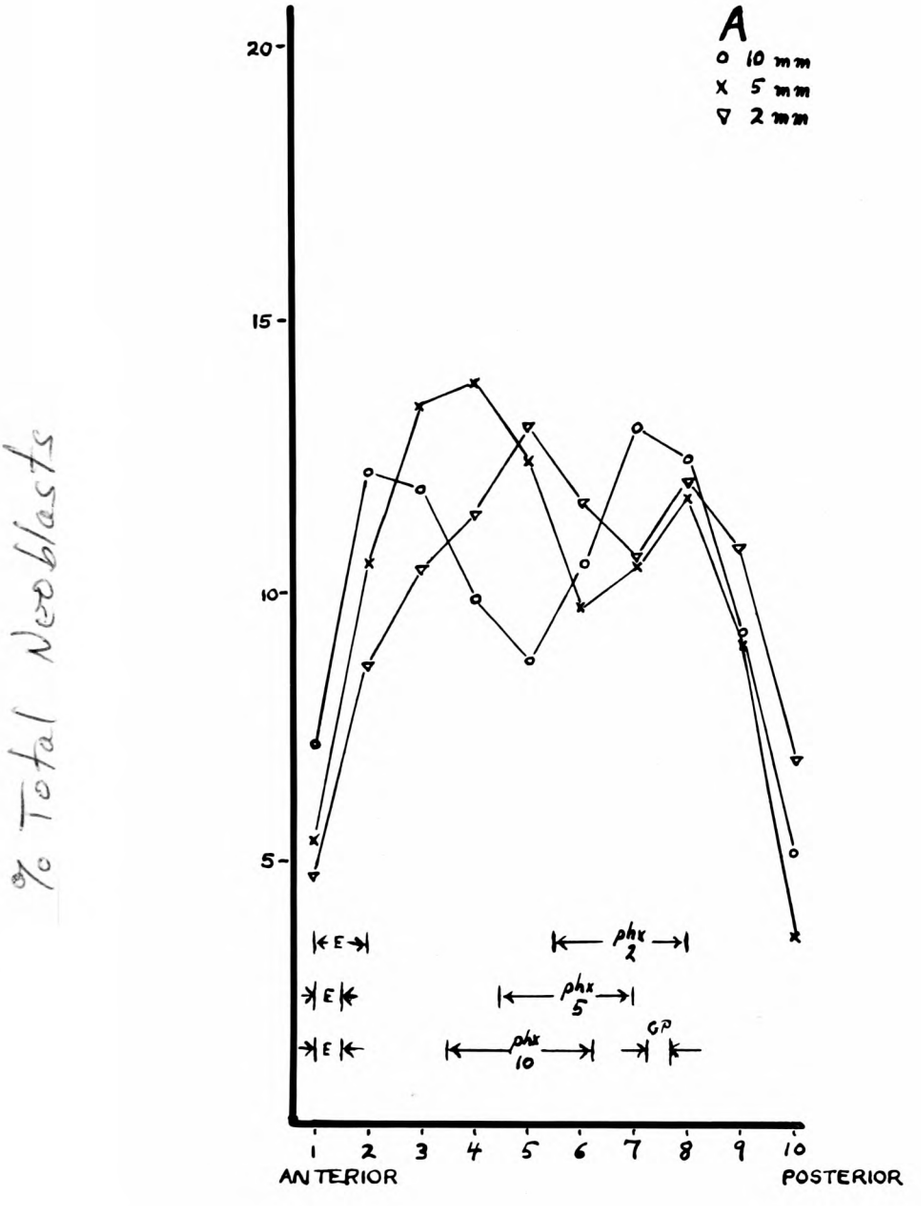

Figure VII-3

Normalized neoblast distribution curves for D. lugubris A

As figure VII-2, but for diploid planarians. The arrows show the anterior and posterior limits of: E = eyes; Phx = pharynx; G.P. = Genital Pore.

pattern of pre- and postpharyngeal maxima with a pharyngeal relative minimum, however, is also found for the diploid.

In order to estimate the possibility that the distribution differences or similarities for various sized animals might be artifacts due to different degrees of shrinkage for different sized animals during fixation and processing, Olmstead and Tukey's corner test for association (1947) was employed. No significant association was found ($P > 0.1$) between size of animal when alive and the ratio of $\frac{\text{fixed}}{\text{live}}$ length (i.e. degree of contraction) for diploids only, triploids only, or the combined data. The mean degree of contraction was 51% with a standard error of $\pm$ 1.5%. Thus it is unlikely that the distributions reported above were influenced by the histological methods employed.

D Corrections for Multiple Counting

The method of Abercrombie (1946) was employed to correct for the possibility of counting a cell in more than one serial section. However, as Abercrombie assumed that errors due to the difference between cell diameter and cell diameter as measured in sections are negligible, a more rigorous formula has been derived and is discussed in Appendix III. For the conditions reported, this correction amounts to only 1%, but for other conditions, this correction need not be negligible. The value of Abercrombie's correction factor for diploids was found to be the same as that for triploids. Thus the relative positions of the points for diploids and triploids (figure

VII-1) remain unchanged, a simple shift of scale giving the true number (lower scale) instead of the absolute count.

E The Volume of a Planarian (fixed state) as a function of its length

The relationship between the length of a triploid planarian and its volume (minus that enclosed by the pharynx sheath) is shown in figure VII-4. The line was fitted by the method of least squares. Analysis of variance shows significant linearity ($p < 0.05$). The regression equation:

$$\log V = 2.63 \log L + 7.33 \text{ (for L in mm, V in } \mu^3)^1$$

has a slope of 2.63 which is highly significantly different from that for the neoblast regression line ($t_{10} = 4.01$; $P < 0.01$). Thus the number of neoblasts in a planarian rises with length at a slower rate than does the parenchymal + gut volume, so that larger animals have a lower density of neoblasts, although more of them.

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1. Similarly, the regression equation for tetraploid D. lugubris was found to be:

$$\log V = 2.85 \log L + 7.42$$

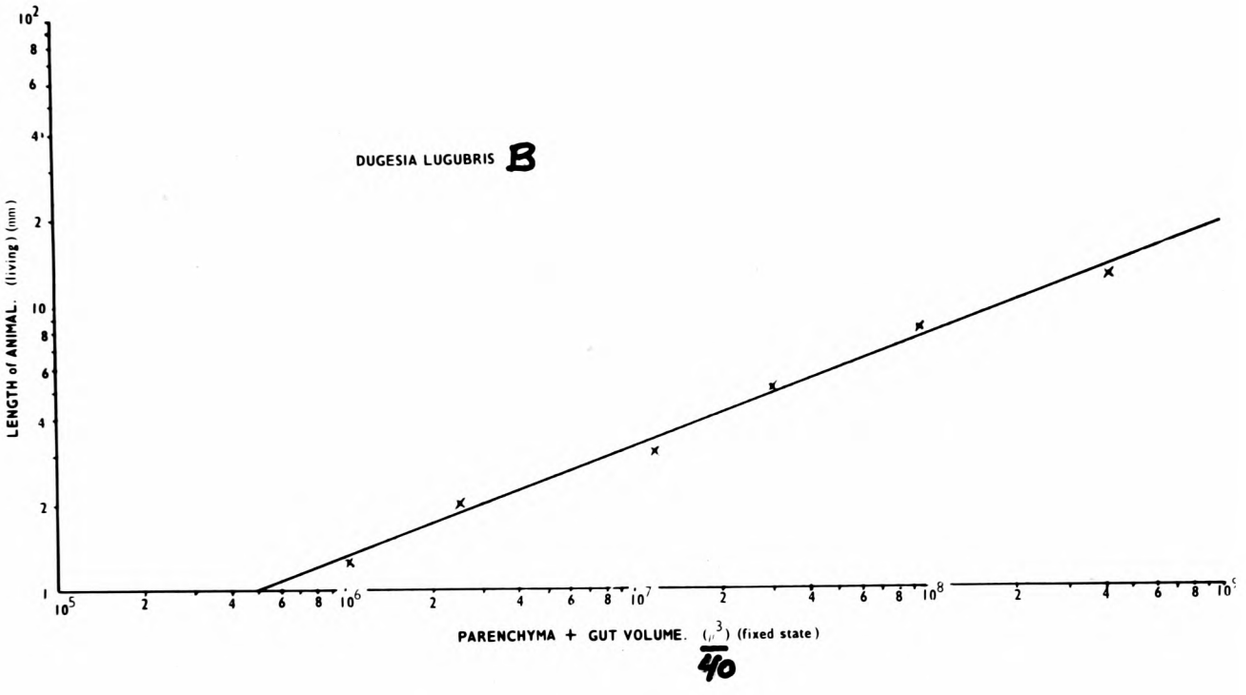

Figure VII-4

Relationship between planarian length and parenchymal plus gut volume

Data points and regression line for parenchyma and gut volume upon length of animal when alive. Each point represents the mean value for two or three animals.

		Anterior 1	2	3	4	5	6	7	8	9	Posterior 10	N_{total}
10mm A	1	7.52	11.38	11.90	9.32	8.09	10.26	12.77	12.28	10.11	6.36	245,210
	2	6.82	12.90	11.79	10.19	9.23	10.01	13.30	12.57	8.32	4.07	239,515
		*→ E ←			←	Phx	→		→ G ←			
5mm A	1	6.25	11.16	13.16	13.97	12.58	9.72	9.94	12.00	8.14	3.06	93,734
	2	4.52	9.97	13.60	13.60	12.27	9.67	11.14	11.31	9.81	4.12	79,717
		→ E ←				←	Phx	→				
2mm A	1	4.44	8.06	9.75	11.10	13.51	12.61	10.98	12.47	10.75	6.33	14,435
	2	5.41	9.38	11.24	12.49	12.15	11.35	10.22	10.69	10.09	6.98	11,082
	3	4.27	8.34	10.32	10.52	13.24	10.93	10.75	12.83	11.31	7.49	10,947
		←E→					←	Phx	→			
2mm A	mean	4.71	8.59	10.44	11.37	12.97	11.63	10.65	12.00	10.72	6.93	
	σ	0.62	0.69	0.75	1.01	0.72	0.87	0.39	1.14	0.61	0.58	
12mm B	1	6.32	10.50	12.07	12.10	13.06	8.36	10.37	11.96	9.49	5.77	327,575
	2	8.62	11.96	11.54	12.32	11.50	9.32	10.47	10.57	8.55	5.16	303,110
	3	7.08	11.54	13.06	11.76	9.25	9.97	12.07	11.40	8.00	4.98	247,517
		→ E ←				←	Phx	→	→ G ←			
12mm B	mean	7.34	11.33	12.22	12.06	11.27	9.22	11.24	11.34	8.68	5.30	
	σ	1.17	0.75	0.77	0.28	1.92	0.81	1.41	0.71	0.75	0.41	
8mm B	1	7.13	9.95	13.07	14.01	12.57	9.32	12.11	9.87	7.46	4.50	123,511
	2	5.47	7.94	11.05	14.36	12.09	10.26	13.20	11.46	8.40	5.71	135,451
		→ E ←				←	Phx	→				
5mm B	1	3.83	10.16	14.24	16.17	14.53	9.98	10.16	11.09	7.05	2.78	76,343
	2	3.77	10.27	12.55	15.37	14.42	10.42	8.13	12.36	8.52	4.19	70,283
		→ E ←				←	Phx	→				
3mm B	1	4.47	9.59	12.45	13.62	13.17	9.52	8.79	12.05	10.75	5.60	29,181
	2	6.40	12.35	15.79	13.72	11.10	8.57	8.44	10.95	8.56	4.11	23,675
		→ E ←				←	Phx	→				
2mm B	1	7.18	10.89	12.58	12.98	11.22	10.04	10.02	12.52	8.82	3.75	7,463
	2	8.43	12.39	13.75	13.34	12.03	10.27	9.19	9.19	6.91	4.49	7,246
		←E→					←	Phx	→			

*E = Eyes. Phx = Pharynx. G = Genital pore.

Chapter VII - Part 2

Discussion of Histological Results

Chapter VII - Part 2

DISCUSSION

Lender and Gabriel (1960) have published a neoblast distribution curve for Dugesia lugubris (presumably of biotype B (triploid), that commonly found in Northern Europe (Benazzi, 1957)) serially sectioned at 5μ. They counted five sections at each of 23 sites along the antero-posterior axis of the animal. Regraphing their data, connecting the points with straight lines and integrating the area under the curve yields as an estimate of the total number (absolute count) of neoblasts in their 8 mm (measured when alive?) animal, 2.00×10^5 cells. Applying Abercrombie's correction $/\overline{5}/(7 + 5) = 41\%/$, gives the corrected total as 8.2×10^4 cells. This is within 13% of our corrected number of neoblasts for an 8 mm triploid (7.3×10^4). The distribution curves presented here differ from that of Lender and Gabriel in that instead of two maxima prepharyngeally, we find one major maximum prepharyngeally and another smaller maximum postpharyngeally. The shape of our distribution curve is more like those of Brøndsted and Brøndsted (1961) for D. lacteum and D. torva. However, quantitatively we differ by an order of magnitude.[1] We did find a small relative maximum just posterior to the eyes as did Lender and Gabriel, but in

1. The Brøndsteds report neoblast totals of $\sim 3 \times 10^4$ for 15 mm animals which should be compared with our estimate of $\sim 3 \times 10^5$ for animals of the same length.

our material this was swamped by the rising prepharyngeal maximum. The distribution curves presented here are also in agreement with the qualitative description of Pedersen (1959b) for Planaria vitta. The fact that our estimate of the total number of neoblasts in Lender and Gabriel's material agrees as well as it does with our own suggests that the differences in the distribution curves might be due to different fixation methods, as the Brøndsteds used Zenker, and we formol-Zenker or Susa, whereas Lender and Gabriel used 80% alcohol or Carnoy. Pedersen (1959b) notes that Carnoy fixation alters the shape of the neoblast. Stéphan-Dubois (1961), using the methods of Lender and Gabriel, studied the neoblast distribution of D. lacteum. The shape of her distribution curves (7-8 sections counted at each of six sites for three animals of ≈10-11 mm length) differs from that of the Brøndsteds for D. lacteum and from those of the present report and that of Lender and Gabriel for D. lugubris. The number of neoblasts per half section, which Stéphan-Dubois publishes, when corrected with Abercrombie's correction (from her photograph of D. lacteum neoblasts, the measured nuclear diameter is ≈5μ) yields (in units of neoblasts per 10μ section), 271 in the region of the brain, 397 postcephallicly but within the first 10% interval, 385 in the third 10% interval, 440 between the fifth and sixth intervals (region of the pharynx), 397 in the seventh interval (postpharyngeal) and 225 in the ninth

interval. The difference between these counts and the present ones for corresponding positions in a 12 mm D. lugubris, do not differ by more than 25% of the sum of the corrected number of neoblasts at each level for both species. Thus the counts of the Brøndsteds and those of Stéphan-Dubois do not agree with each other. This difference is difficult to reconcile in view of the fact that the total counts obtained by Brøndsted and . Brøndsted for D. lacteum and D. torva (3.78×10^4 and 3.13×10^4, respectively) of 15 mm live length, are almost an order of magnitude lower than the extrapolated value for D. lugubris (figure VII-1). Also, their method of correcting for double counting would tend to give values higher, rather than lower, than the actual number, thus diminishing, rather than enhancing, this difference.

The constancy of the relative position of the pharynx in triploids from 2 mm (just hatched) to 12 mm (cocoon-laying adult) in length as opposed to the shift forward in the diploids is unlikely to be a fixation artifact as planarians of all sizes (for the fixatives used) shrank to the same percentage of their live length. This implies a difference in mode of growth between diploids and triploids. It would seem that triploids grow uniformly, whereas diploids grow more from the postpharyngeal than from the prepharyngeal end. The diploid pharynx shift was also observed in animals not used for the neoblast counts.

The observation that the volume of D. lugubris B (excluding that of the pharynx, although its inclusion would not alter the conclusion) increases with length at a faster rate than does the total number of neoblasts, implies that the overall density of neoblasts in a large animal is lower than that in a small animal. This could have some bearing on the problem of senescence in planarians. A large animal is physiologically "old" with respect to regeneration ability (head-frequency), whereas, if it is starved or cut in half to regenerate, the reproportioned individual (after completion of morphallaxis) becomes physiologically "young".

The distribution curves for fixed total volume were, in general, uniform in pattern, rising smoothly to a maximum in the fifth 10% interval and then dropping caudally.

The distribution curves of the ratio of $\frac{\%\ \text{Neoblasts}}{\%\ \text{volume}}$ for each 10% interval along the antero-posterior axis were rather variable but had a general pattern. In the 5, 8, and 12 mm long triploids, the ratio for the head (1st 10% unit) was 1.0, increasing with length from 1.14 (5 mm animal) to 1.75 (12 mm animal). The ratio decreases posteriorwards from the first interval, is a minimum in the fifth or sixth interval (0.72 - 0.78), then rises until the eighth interval, and finally falls for the 5 mm animal, levels off for the 12 mm animal and continues rising for the 8 mm animal.

Reynoldson (1961) found that newly-hatched D. lugubris

(2.5 mm long) grow, when fed once a week under laboratory conditions, at a rate of 2.5 mm per four weeks, and shrink, on starvation at a summer temperature, at a rate of 2.0 mm per four weeks. As Lender and Gabriel (1961) state that in two months-starved D. lugubris (reduced to two thirds of their length) the shrinkage is not due to the loss of the neoblasts, it would be interesting to see quantitative data as to whether or not the number of neoblasts in a starved animal is, in fact, the same as the number that it would have had, had it not been starved.

This chapter has presented estimates of the numbers of neoblasts in the preirradiation experimental animals, which is one of the parameters of the model which translates animal survival into cellular survival.

Since the total number of neoblasts per animal is a function of animal length, and since animals of constant size were selected for irradiation experiments, the value of N appropriate to the other data to be used in the model is (for D. lugubris):

$N_o = 1.406 \times 10^5$ upper limit - 1.986×10^5 (+ 41% of median value)
lower limit - 9.18×10^4 (- 35% of median value)

The error introduced by deviations from the regression line was negligible compared to that resulting from the variation in animal size. If we take the range to be equivalent to

approximately $\pm$ 2 S.E., then the standard error of N_o is approximately $\pm$ 0.267 x 10^5 or $\approx$ 20%.

Chapter VIII

Grafting Methods and Results

Chapter VIII

Grafting Methods and Results

In chapter VI α_{50} was defined as the probability that a single surviving neoblast could repopulate the animal to produce a given probability of survival (50%). The reciprocal of α is, then, the number of neoblasts necessary to produce this given survival probability. $N_0\alpha$ is the proportional excess of neoblasts above the minimum, necessary for the above-chosen survival probability. Thus if all of the neoblasts in a given animal are sterilized by radiation, and a defined number of neoblasts are grafted into such a host, that graft size which yields 50% survival should contain approximately $1/\alpha_{50}$ neoblasts.

A Grafting Technique

The grafting technique was a modified version of that of Dubois (1949). For optimum results, the room should be cool (10-15°C). As our laboratory is built on the "open plan", these temperatures were achieved by the use of a hood, the inside of which was cooled by blocks of dry ice. P.V.C. sheets were used as curtains to restrict the exchange of air between the hood front and the room. Figure VIII-1 shows the grafting area. The supralethally irradiated (10 k rad) hosts and the unirradiated donors were placed in 10^{-4} (w/v)

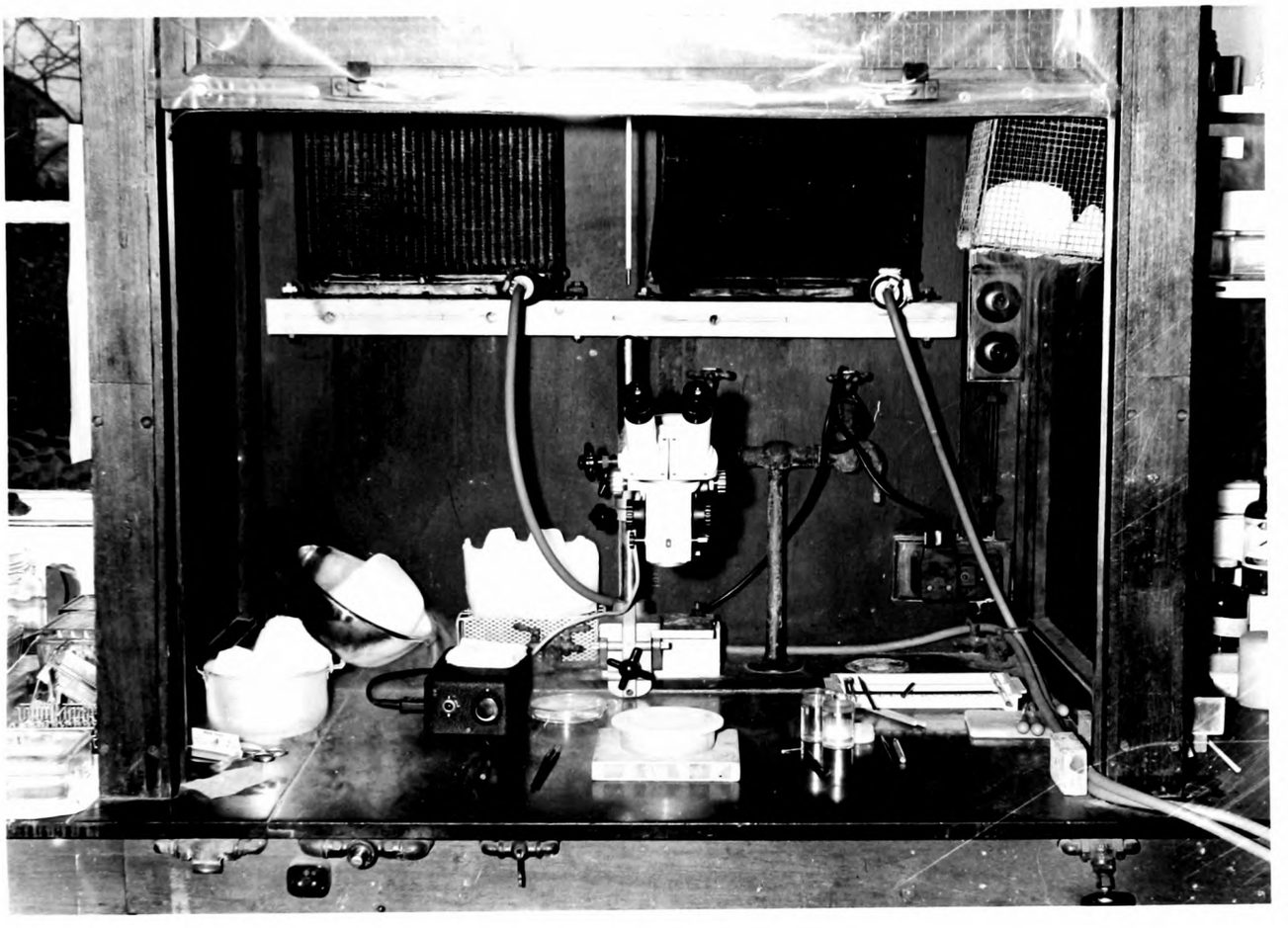

Figure VIII-1

The grafting area

P. V. C. insulation sheets are tacked up to give a better view.

nicotine sulphate solutions for a minimum of 25-30 minutes. This treatment resulted in the contraction and immobilization of the animals. Once immobilized, two hosts and one donor were placed on an ice cold parafin block (figures VIII-2 and VIII-3) and all were decapitated with a razor-sharp miniature chisel (figure VIII-4). The decapitation served the two-fold purpose of making the animal less reactive to the subsequent cuttings of the operation, and provided a wound surface some distance away from the implantation site of the graft, so that neoblast migration was encouraged. As the major cause of graft failure was the planarian's covering of its wound (cut) surfaces with mucus before the graft and host cut surfaces could be brought into contact, the following part of the technique had to be done quickly (2-3 minutes). For a 1 x 1 mm graft, a 1 mm wide (blade width) chisel is taken. Using sharp vertically downward strokes, and wiping the blade clean after each stroke, cuts were made in the host as shown in figure VIII-5, and the excised block of tissue removed. As the host contracts somewhat, after the excision, the excised piece must be made a bit larger than 1 mm x 1 mm. Using two wet paintbrushes (sable hair # 0 and #1) the host was removed from the block and placed dorsum up, on a piece of wet cigarette paper on a nylon drum, the surface of which was common to the miniscus of the 8-10°C water underneath it (q.v.

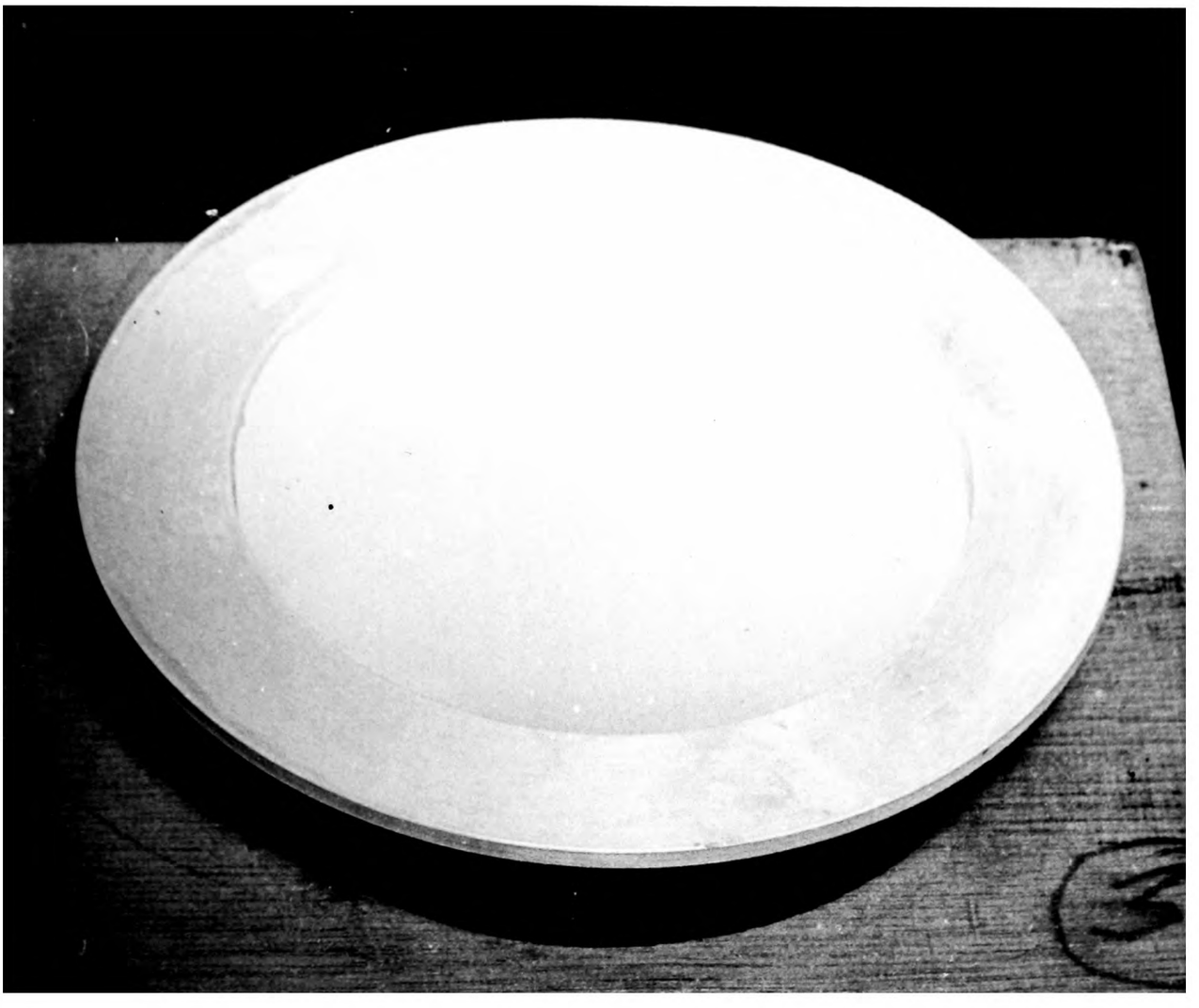

Figure VIII-2

Cooled Parafin block used as "operating table"

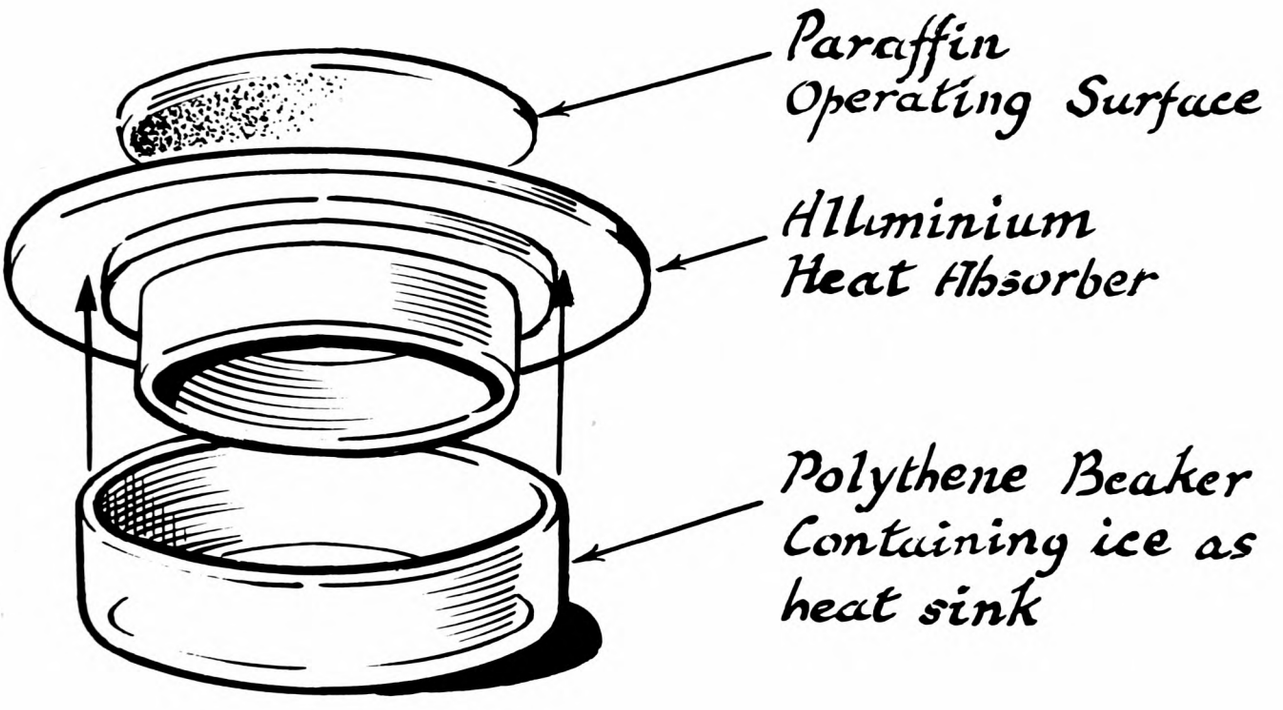

Figure VIII-3

Plan of cooling unit for parafin block
"operating table"

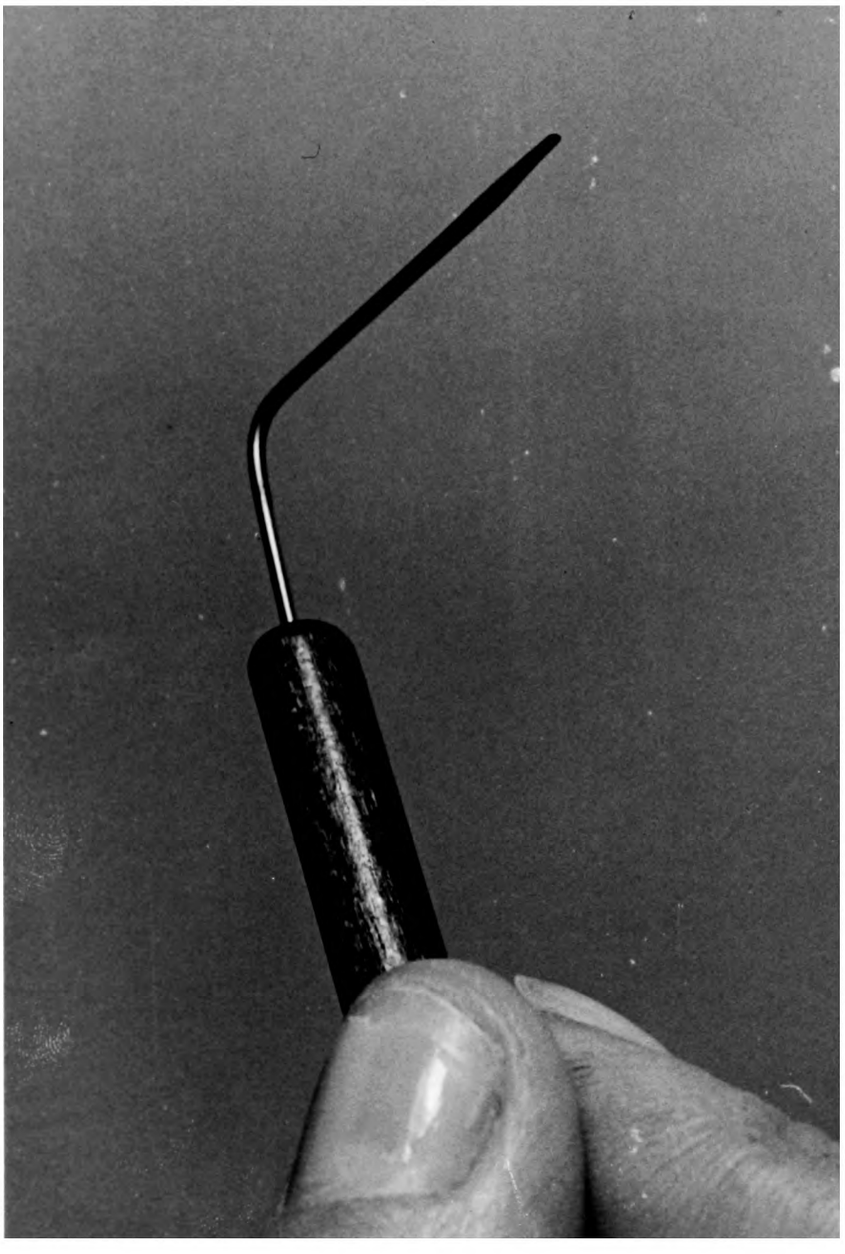

Figure VIII-4

One of the chisels used for grafting operations

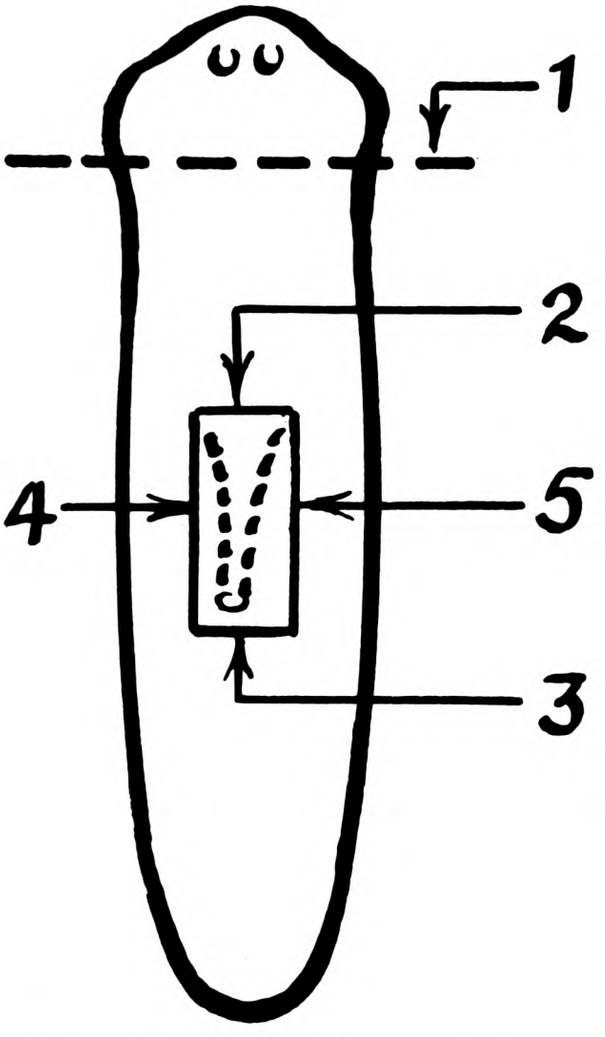

Figure VIII-5

Plan of cuts made to prepare host for graft

figure VIII-6). The purpose of the nylon drum was to keep the planarian damp but at the same time not provide enough water to

(1) speed the rate of dilution of the nicotine sulphate from the planarian tissues,

or (2) allow the planarian to swim about before the graft and host surfaces have had a chance to fuse.

The 1 mm chisel was then used to cut out a 1 mm x 1 mm graft from the donor as shown in figure VIII-7a or b, and the graft was lifted with a very fine artists' paint brush (sable hair #00) and a loop of hair (figure VIII-8). The tip of the paint brush was used to widen the hole in the host and the graft, which was sitting just a little distance from the tip of the paint brush, was slid down the brush and into the hole in the host. The loop of hair was then used to push the host and graft surfaces into contact, and to remove small amounts of mucus if necessary. A lid was placed on the drum containing tube which was then placed in a 8-10°C waterbath to rest undisturbed for 3-4 hours. After 2-3 hours, the graft and host tissues had fused (if they were going to do so at all). Even after 4 hours the fused surfaces were still only weakly attached, however, so great care had to be taken in handling the animals at that stage. The effects of the nicotine sulphate had worn off by that time and the animals, if left any



Figure VIII-6

The drum and paper used as a bed for the host and the freshly placed graft

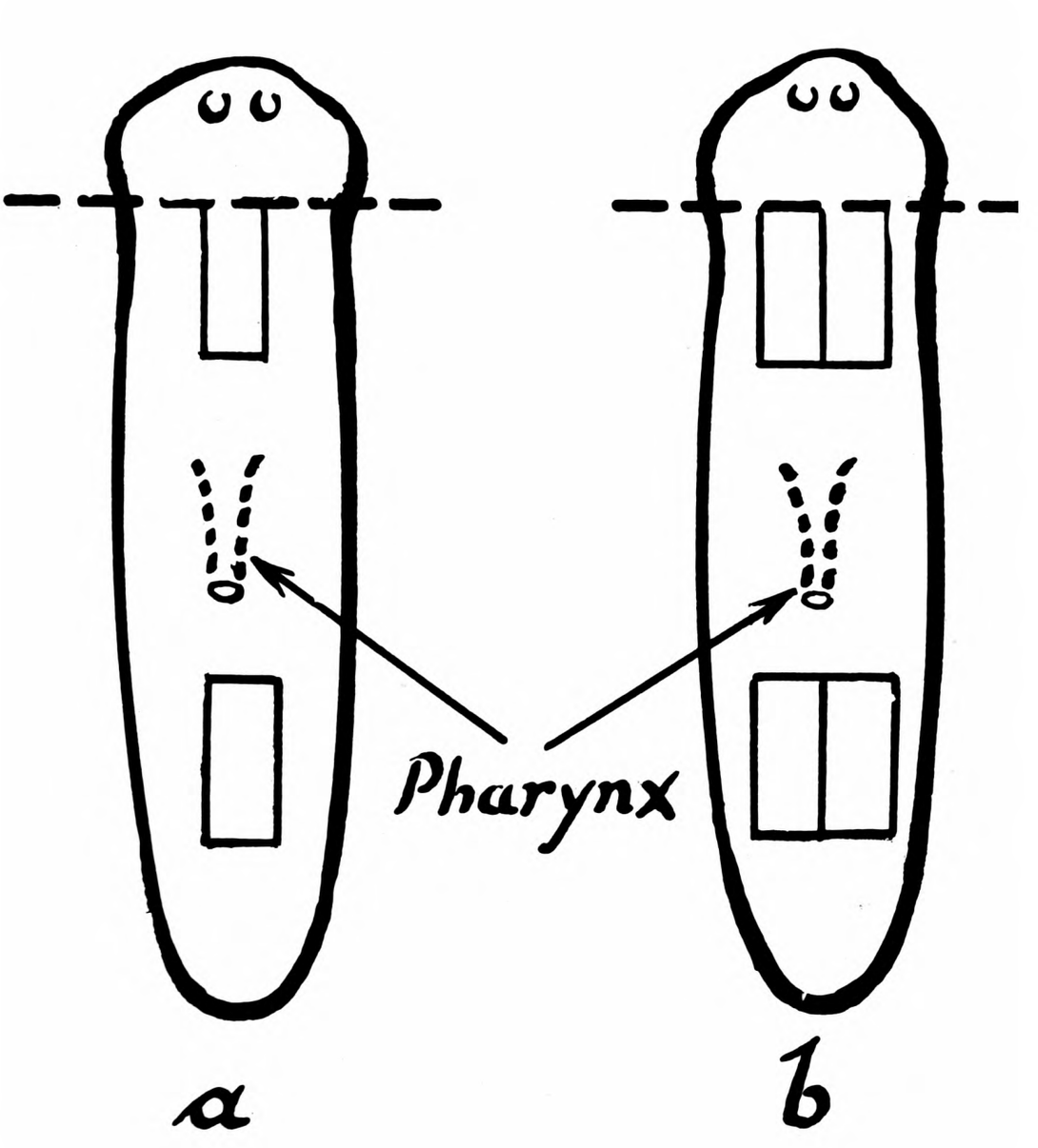

Figure VIII-7

Plan of cuts made to obtain standard grafts

(a) 2 hosts per donor

(b) 4 hosts per donor.

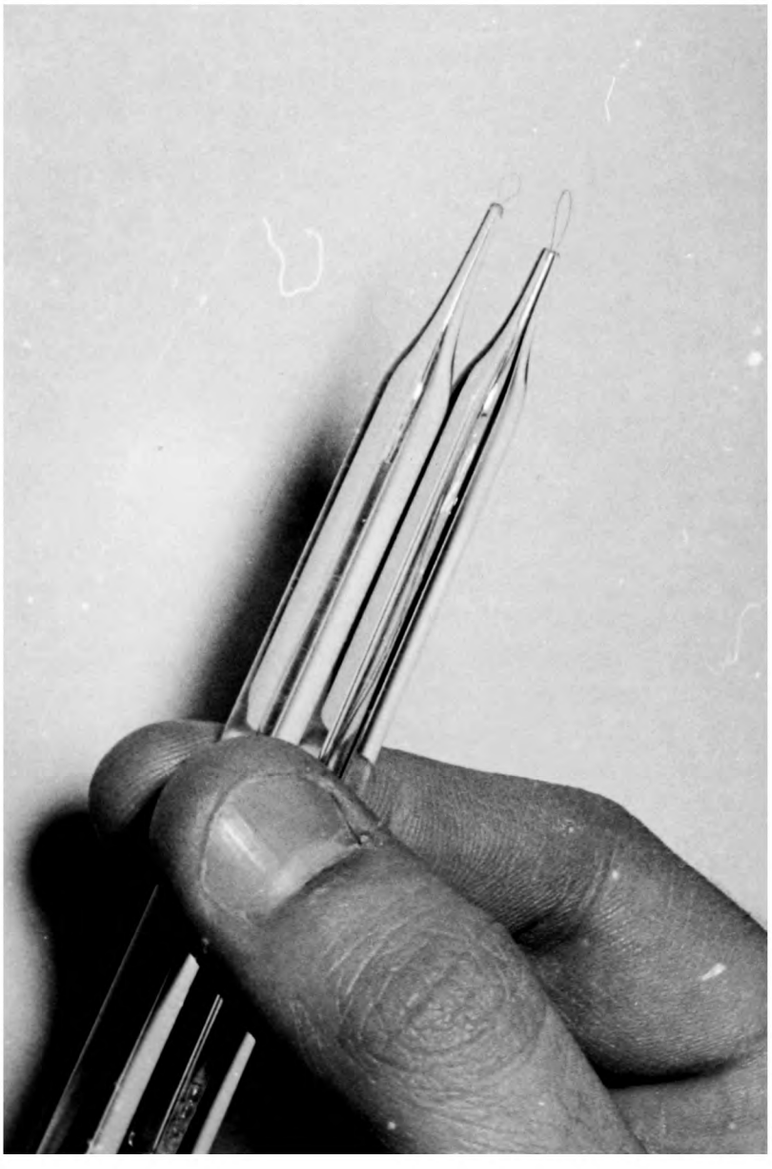

Figure VIII-8

Hair loop used to manipulate graft and host tissues

longer above the surface of the water, would have started to move by muscular contractions which could have dislodged the graft. Therefore, the cigarette paper, with the planarian on it, was lifted with a pair of foreceps and placed into a 13 ml polystyrene tube of mains water. In this environment, if the planarian moved, it was more likely to propel itself by means of its cilia - a uniform motion less likely to have affected the graft adversely. The tube was capped and placed in the 20°C incubator. Two or three days later the paper was removed from the tube and the state of the graft noted. Thereafter, the animal was examined thrice weekly as in most other experiments.

The chisels were made by heating a dissecting needle in a gas flame until it became cherry red, and then hammering the tip flat until the desired width was attained. The needle was then retempered, ground on a wheel, and the shaft bent to an angle convenient for making vertical strokes. A final sharpening was obtained by polishing the blade on an extra fine Belgian sharpening stone. The chisels were repolished after every grafting session (20 hosts).

The degree of success depends very much on the planarian population used, and Stéphan-Dubois (1966, personal communication) who obtained 98-100% takes with the D. lugubris B of Strasbourg found much less success with the D. lugubris B of Nancy. It is of particular interest that aseptic technique

is of no particular advantage for planarian grafting (Brøndsted, 1939) and that interspecific grafting is possible (Santos, 1931; Lindh, 1959).

B Injection Technique

In an attempt to develop an assay technique for reproductively intact neoblasts, similar to that of Hewitt and Wilson (1959) for mouse leukaemia, the following techniques were developed.

Jacobson and Jacobson (1963) in an unofficial report to the Worm Runner's Digest described a technique for the injection of macromolecules, such as RNA, into the planarian gut. It is known that no digestion takes place within the lumen of the planarian gut (Rosenbaum and Rolon, 1960; Jennings, 1957) and that cells and macromolecules are incorporated into the planarian via phagocytosis by the gut columnar cells. In the hope that neoblasts might be able to penetrate the gastrodermis intact, the apparatus shown in figure VIII-9 was built. It consists of a micrometer syringe (Agla) with a Hamilton 35 gauge (127μ O.D.) stainless steel needle, mounted onto a ball socket which in turn sits on a sliding table. The planarian is placed ventrum up in a groove cut in a parafin block which is in thermal contact with a large block of ice. The low temperature anaesthetises the planarian sufficiently so that it does not move during the injection. The needle is inserted into the mouth and up the lumen of the pharynx (which is

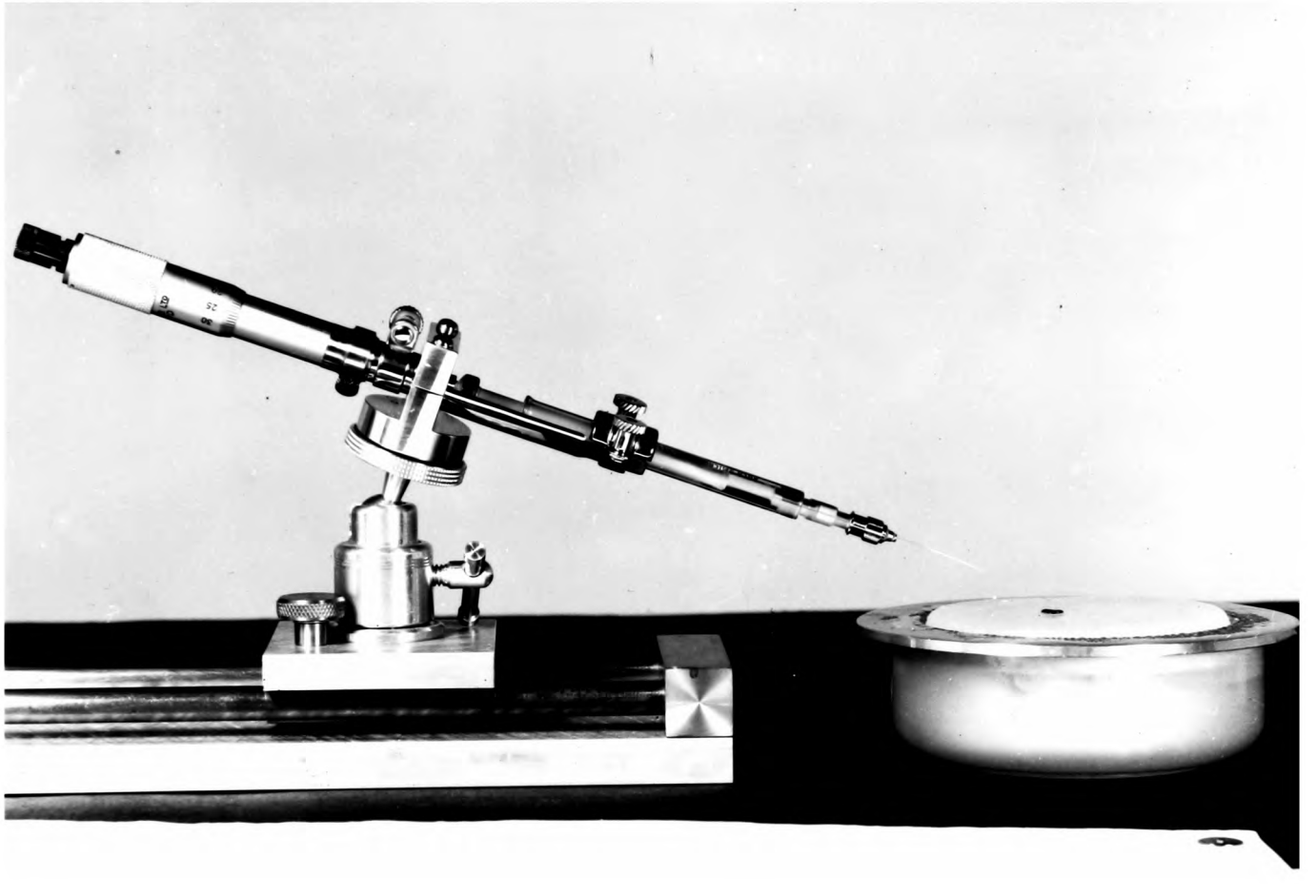

Figure VIII-9

The planarian injection apparatus

130 μ I.D.). The ball socket and sliding table are locked in position and then the micrometer can be used to inject a known volume of fluid (cell suspension). After the injection, when the needle is withdrawn, the injected material remains in the gut. Material injected directly into the parenchyma is eliminated by the planarian (by contraction) as the needle is withdrawn.

The cell suspension was made by taking 10 animals (10-12 mm long), putting them into 0.5 ml of a 1/10 dilution of Holtfreter's in double glass distilled water with 0.004% phenol red as pH indicator. The planarians were drawn into a cell suspension by sucking them up and down in a 1 ml plastic syringe through a No.1 serum needle, then a No.1 hypodermic needle (Steriseal) and finally a No.18 hypodermic needle. The resulting suspension was gently centrifuged through a 25 μ pore nylon bolting cloth[1], and drawn up into the siliconized glass barrel of the micrometer syringe. An aliquot was dispensed from the needle for cell counting on a haemocytometer. The cells appeared to be in good condition on the haemocytometer slide. Yields varied between 10^6 and 10^7 cells/ml and 10-30 μ l could be injected into the gut without subsequent loss of fluid. Thus somewhere between

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1. 25TII "St. Martins" Nylon, Henry Simon Ltd., Cheadle Heath, Stockport, England.

10^4 and 10^5 cells could be injected into the planarian by this method. What proportion of these cells are neoblasts is not known, but as the neoblasts are small relative to the other planarial cells, and are of the order of $<20\mu$ diameter (in vivo, obtained by multiplying fixed diameter x shrinkage factor) some enrichment might be expected. However, injection of 46 lethally irradiated planarians with cells did not improve survival above that of the saline injected (10) or uninjected (38) controls. Presumably the cells did not get through the gastrodermis intact (or were damaged by the suspension technique).

In an effort to determine isotonicity for the planarian cell, specimens of D. tigrina were suspended in sucrose solutions of 10^{-2}M down to 10^{-6}M. Cell and nuclear diameters were measured at 400 magnifications under phase contrast with an ocular micrometer. As a control value, a planarian was squashed in its own fluids and diameters were measured. The measurements which came closest to the control value were those for 10^{-3} - 10^{-4}M with 10^{-2}M having its mean diameter just a bit more than the lowest extreme for the control, and 10^{-5}M having its mean diameter just a bit less than the highest extreme for the control. At 10^{-6}M there was much debris and cells could be seen to continue to expand and burst. Figure VIII-10 shows the mean values for the cell diameters.

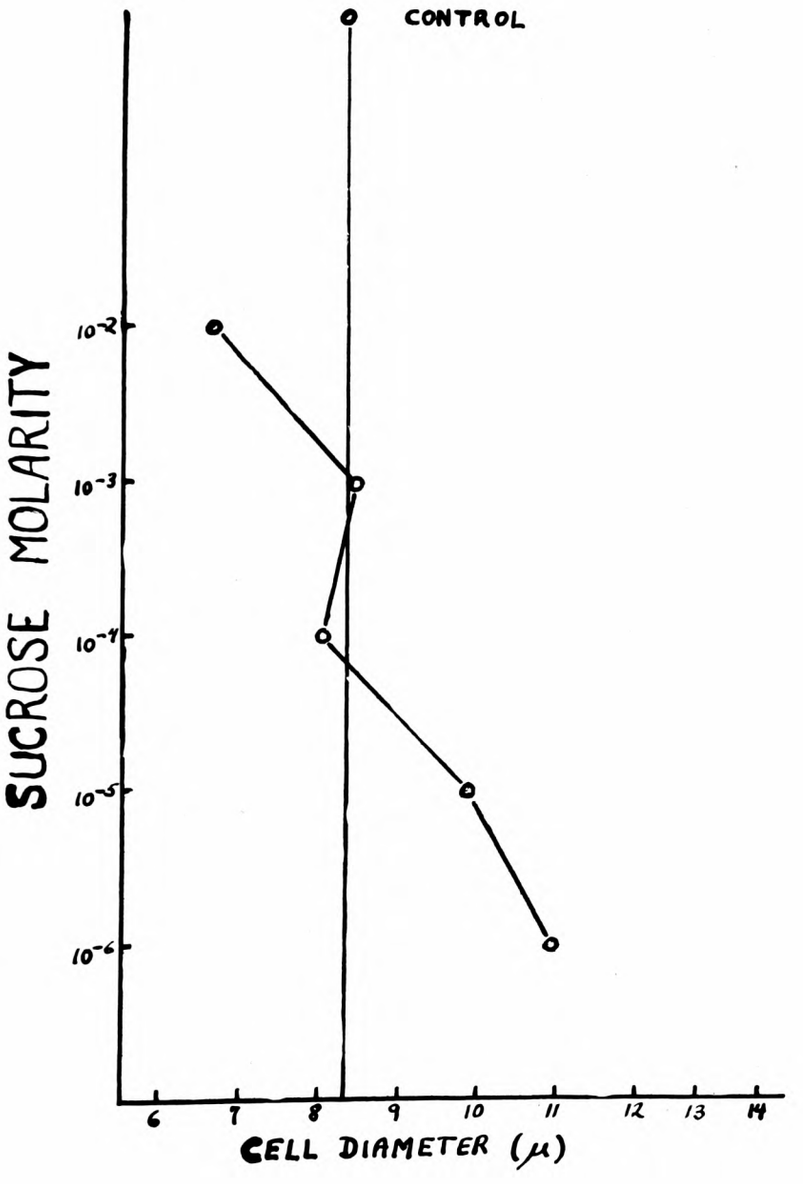

Figure VIII-10

Diameters of planarian cells in sucrose suspensions (D. tigrina)

C Results of Grafting Experiments

1 Diploid→Diploid Grafts

In these experiments an attempt was made to graft 1 x 1 mm or 1 x 2 mm blocks of tissue from 80 donors into 339 supralethally irradiated (10 krad) hosts. Of the 100 1 x 1 mm grafts, 35 were defined as successful (i.e. fused to host on third day after graft placement) and of these, 18 survived the 60 day postirradiation endpoint time. Of the 90 1 x 2 mm grafts, only 6 were successful, yielding 5 survivors. The combined yield of successful grafts was 21.6%. Of the 149 unsuccessfully grafted hosts, only three survived. (It may be possible that some neoblasts were transferred during the short time that the graft remained in contact with the host tissues?) A summary of the results of the grafting experiments used to determine the value of α are presented in table VIII-1, and the graph of cell dose (No. neoblasts per graft) vs. % survivors is shown in figure VIII-11. The value of $1/\alpha_{50}$ for diploid D. lugubris as determined by these experiments was found to be $(1.80 + 0.27/-0.33) \times 10^4$, so that $\alpha_{50} = (5.56 + 0.73/-1.25) \times 10^{-5}$.

A second method of measuring α was also employed. Parts of animals were radiation sterilized, while the remaining portions were shielded (q.v. chapter IV B for methods and chapter IX for results). Shielding of 18% ± 5% of the body length (from either anterior or posterior end) yielded 50%

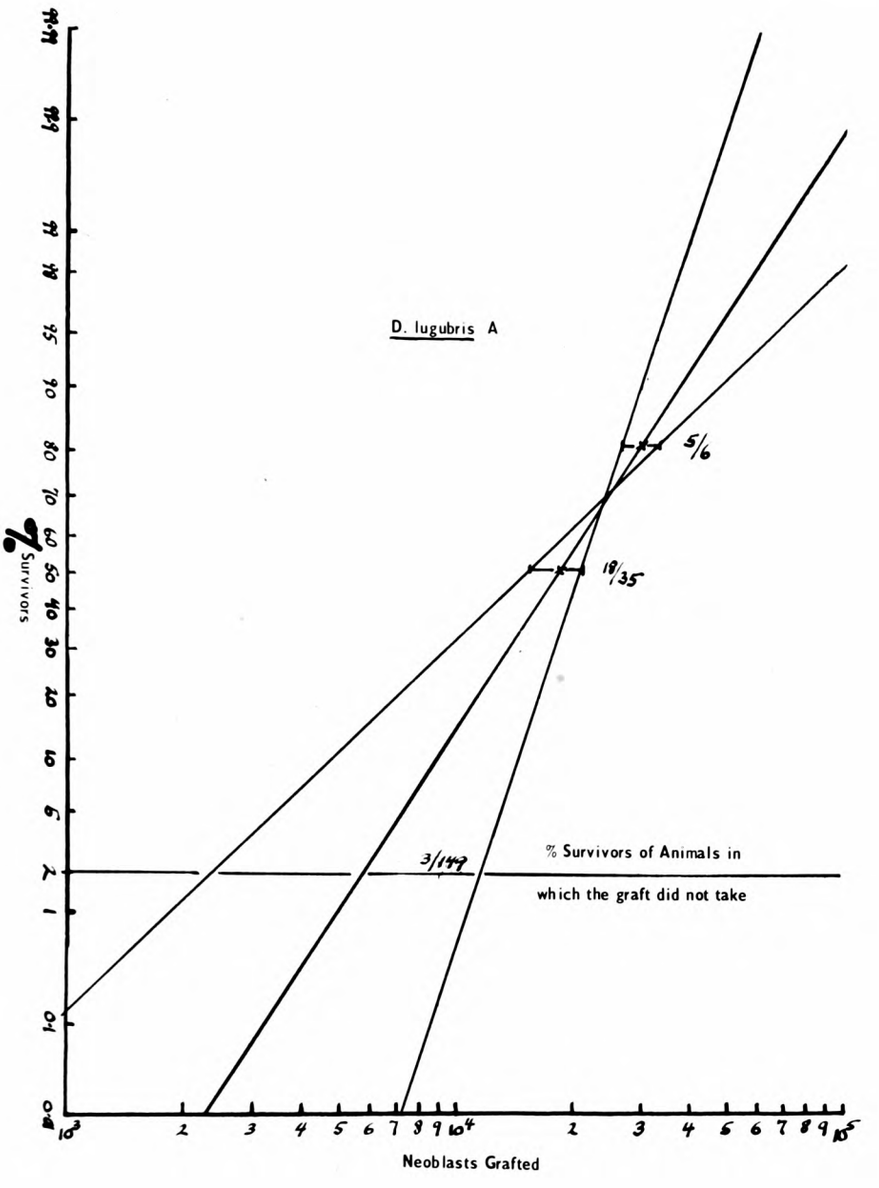

Figure VIII-11

Effect of neoblast graft dose on % survivors (D. lugubris A)

The fractions represent the number of survivors (numerator) divided by the number of takes (denominator).

survivors (figure VIII-12). A piece of planarian, containing either the anterior or posterior extremity, and having a length of 18% of the length of the entire animal, contains on the average 15% ± 5% of the total number of neoblasts in the intact animal. A graph illustrating the discrepancy between % body length and % total neoblasts (based on 8-12 mm diploid and triploid animals, averaged for distribution differences between anterior and posterior portions of the animal) is presented in figure VIII-13. Therefore the number of neoblasts necessary for 50% probability of survival, $1/\alpha_{50} = (0.15 \pm 0.05)N_o = (2.10 \pm 0.07) \times 10^4$ cells, and $\alpha_{50} = (4.76 \pm 0.16) \times 10^{-5}$ - a slightly but not significantly lower value than that obtained from the grafting experiments.

It is interesting that the values of α derived by these two different methods are in reasonable agreement, although some of the neoblasts in the grafts were likely to have been used to help fuse the graft to the host. However, the cutting and decapitation of the hosts performed in the grafting procedure should also have given a greater stimulus for neoblast migration from the graft into the depopulated tissues. Thus the magnitude of each of these effects is small, or nearly equal and opposite in effect so that they cancel each other out.

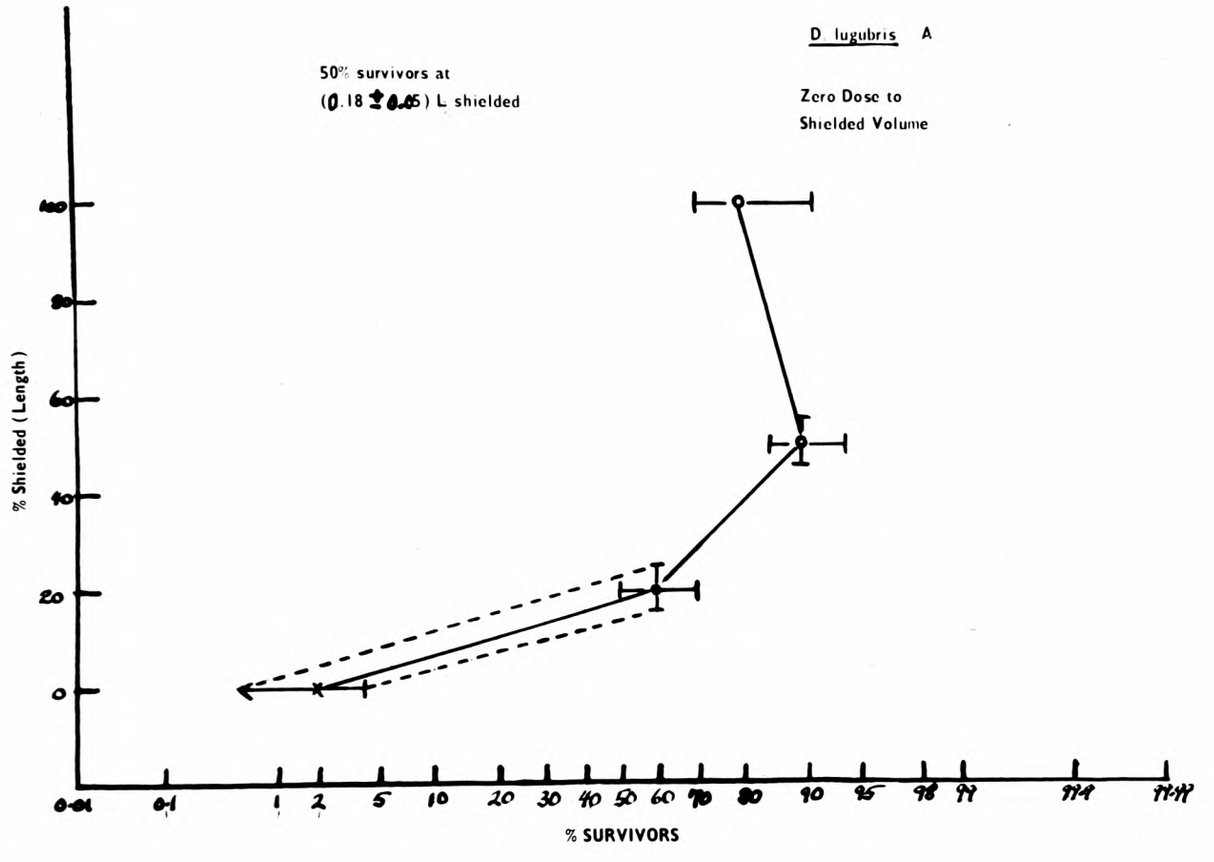

Figure VIII-12

Relationship between survival probability and % length shielded (D. lugubris A)

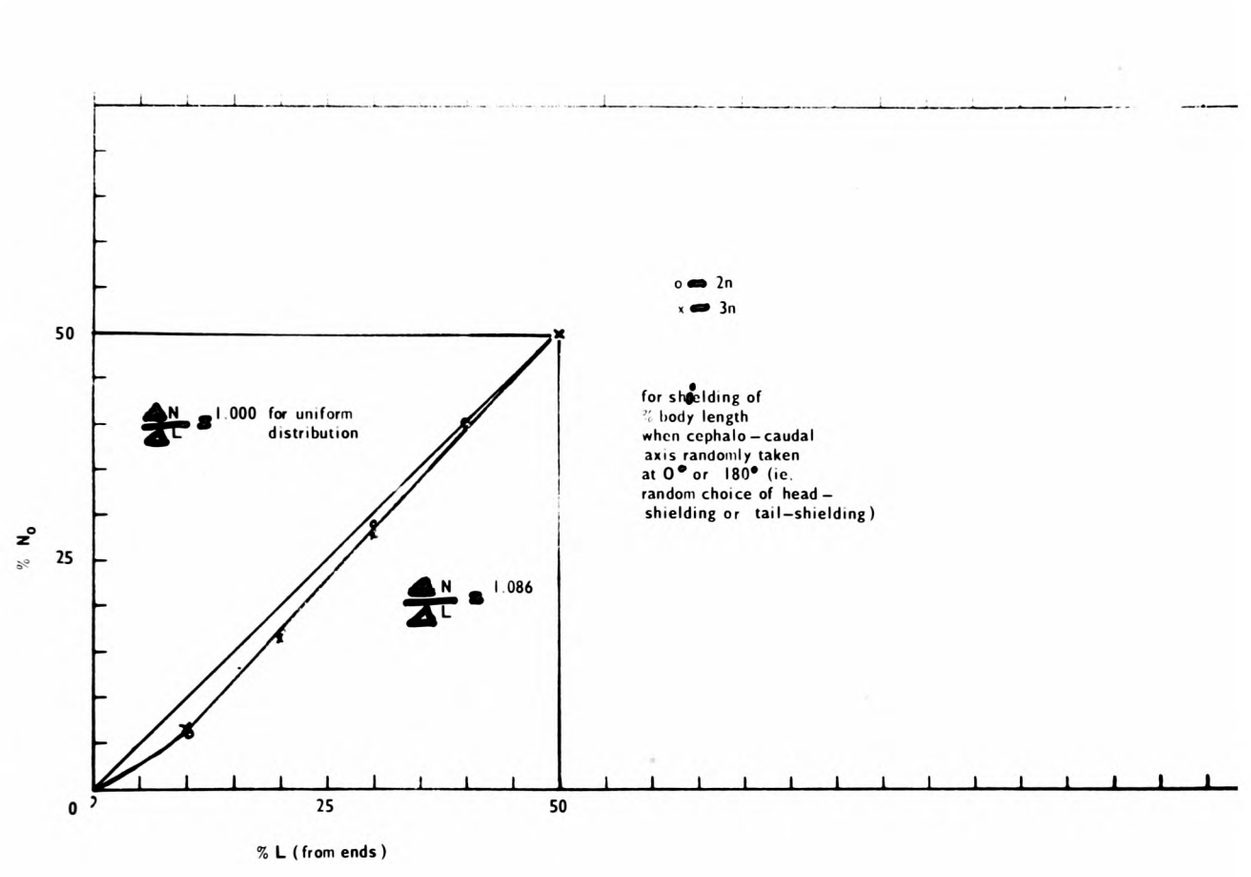

Figure VIII-13

Relationship between % length shielded and % neoblasts in the shielded region (^{90}Sr β^- irradiation)

2 Triploid→Triploid Grafts

Five sizes of graft, cut according to the 2 or 4 hosts per donor scheme (q.v. chapter VIIIA), made it possible to transplant ten different doses of neoblasts. Of the 589 hosts into which grafts of 0.25, 0.5, 1.0, 1.5, or 2.0 mm^2 (length x width, thickness approximately constant), were placed, only 89 (15.1%) were successful. No simple relationship could be found either between graft size and % take (q.v. figure VIII-14) or between the numbers of neoblasts in a successful graft and subsequent host survival (figure VIII-15). Thus the grafting experiments were not suitable for estimation of α for the triploid animals.

However, on the basis of shielding experiments (q.v. previous section and chapter X), an estimate of α for the triploid animals was obtained. Shielding of 17% ± 5% of the body length, i.e. $(0.15 \pm 0.05)N_0$, (q.v. figures VIII-13 and 16) gave 50% survivors. Thus $1/\alpha_{50} = (1.96 \pm 0.07) \times 10^4$ and $\alpha_{50} = (5.10 \pm 0.18) \times 10^{-5}$.

This value for α_{50} obtained for triploid D. lugubris is not significantly different from either of the α_{50}'s determined for diploid D. lugubris. This suggests that the probability of a single surviving neoblast repopulating the animal is not affected by ploidy differences.

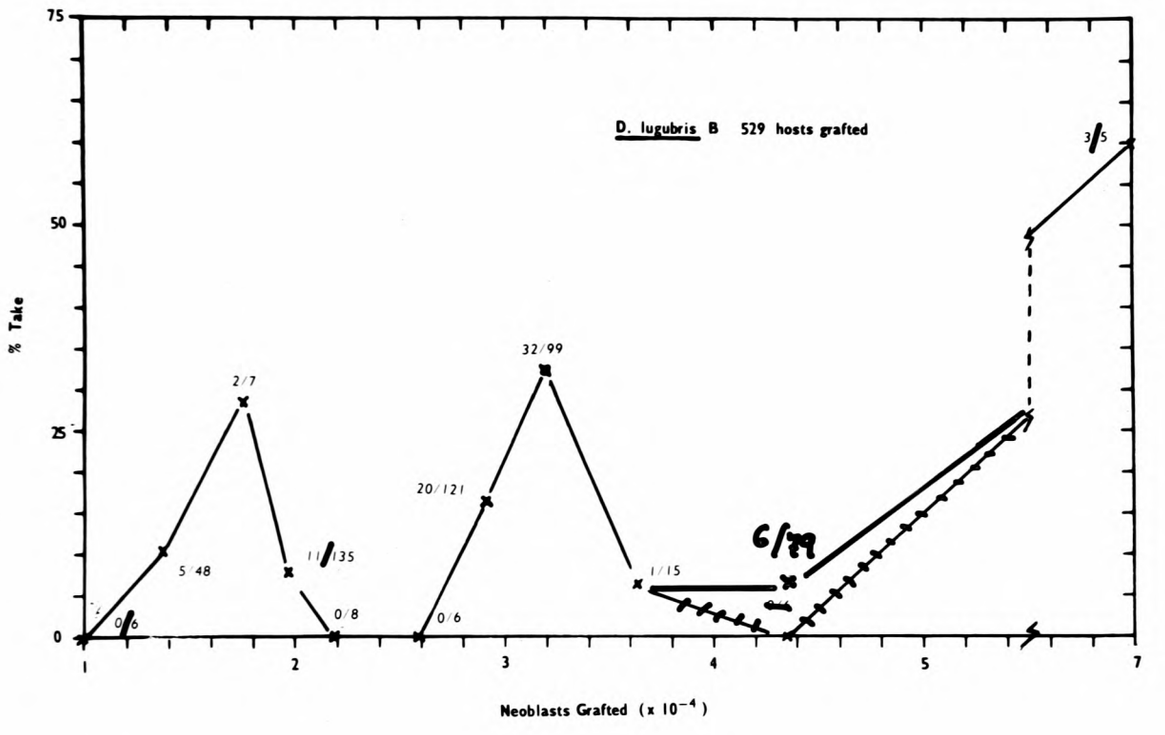

Figure VIII-14

Relationship between graft size and % take (D. lugubris B)

The fractions represent the number of takes (numerator) divided by the number of grafts placed (denominator).

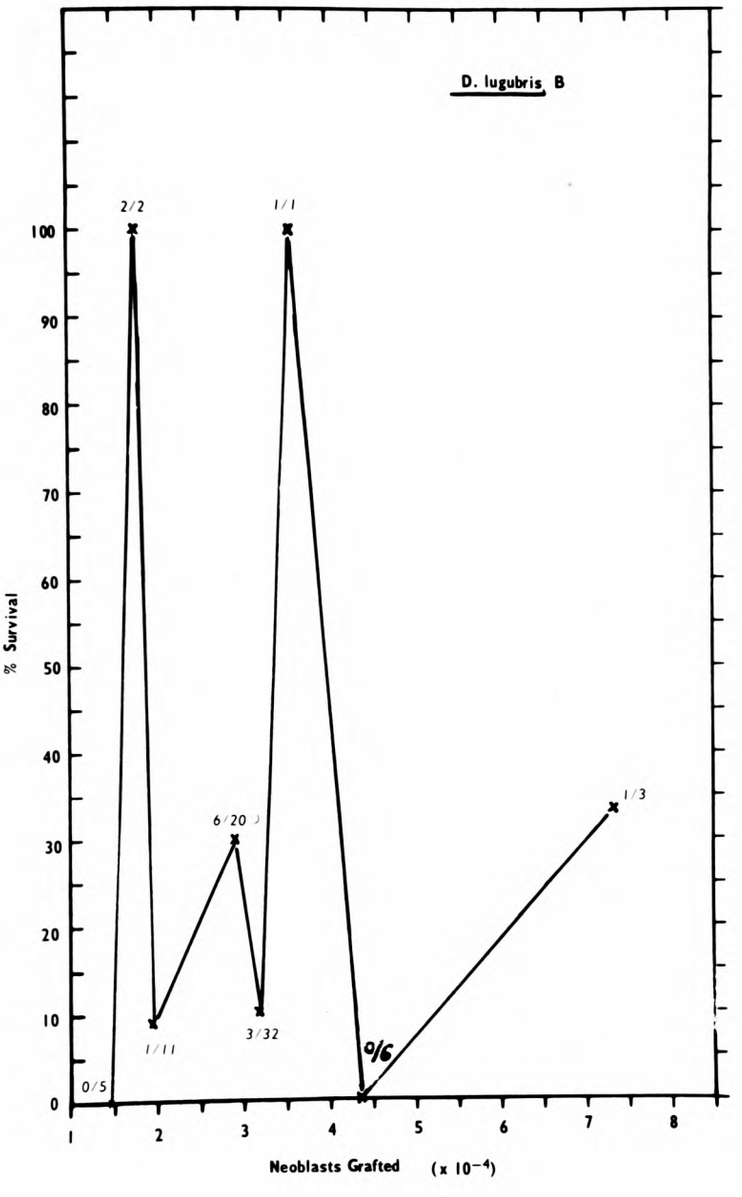

Figure VIII-15

Relationship between neoblast graft dose and % survivors (D. lugubris B).

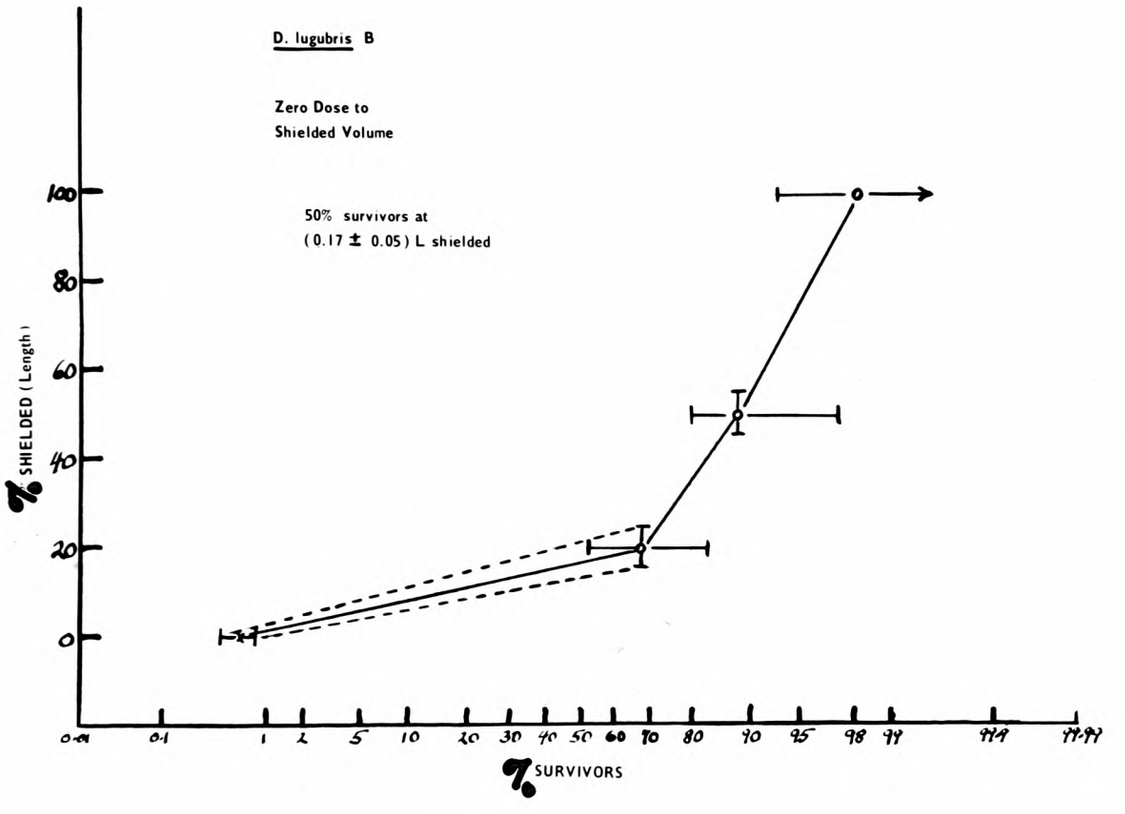

Figure VIII-16

Relationship between survival probability and % length shielded (D. lugubris B)

Table VIII-1

Results of Grafting Experiments

Biotype	Graft Size (mm)	Graft Type	N_g* ± S.E.	No. Attempted	No. Successful	No. Survivors	% Survivors	% Take	% N.T. Survivors
A	1 x 1	4H/D	(1.85 ± 0.27)x10^4	100	35	18	51.5	41/190 = 21.6%	3/149 = 2%
"	1 x 2	4H/D	(3.01 ± 0.34)x10^4**	90	6	5	83.5		
"	1 x 1	2H/D	(2.06 ± 0.22)x10^4	-	-	-	-		
B	0.5 x 0.5	4H/D	(1.00 ± 0.11)x10^4**	6	0	-	-	89/589 = 15.1%	3/500 = 0.6%
"	1 x 0.5	4H/D	(1.36 ± 0.15)x10^4**	48	5	0	0		
"	0.5 x 0.5	2H/D	(1.75 ± 0.20)x10^4**	7	2	2	100		
"	1 x 1	4H/D	(1.96 ± 0.04)x10^4	135	11	1			
"	1 x 0.5	2H/D	(2.18 ± 0.25)x10^4**	8	0	-	-		
"	1 x 1.5	4H/D	(2.58 ± 0.29)x10^4**	6	0	-	-		
"	1 x 1	2H/D	(2.90 ± 0.10)x10^4	121	20	6	30		
"	1 x 2	4H/D	(3.19 ± 0.54)x10^4	99	32	3			
"	1 x 1.5	2H/D	(3.62 ± 0.41)x10^4**	15	1	1	100		
"	1 x 2	2H/D	(4.34 ± 0.89)x10^4	79	6	0	0		
"	2 x 2	2H/D	(7.15 ± 0.82)x10^4**	5	3	1	33		
"	1 x 1 500 rad	4H/D	(1.96 ± 0.04)x10^4	20	4	1	25		
"	1 x 1 540 rad	2H/D	(2.90 ± 0.10)x10^4	20	2	2	100		
"	1 x 1 250 rad	2H/D	(2.90 ± 0.10)x10^4	20	3	0	0		

* Number of neoblasts per graft ± S.E.

** Estimated by interpolation or extrapolation of measured values. Standard errors estimated to be ~11.4% as this was the mean % standard error for the measured values.

Chapter IX

Calculation of Extrapolation Numbers

Chapter IX

Calculation of Extrapolation Numbers

Previous chapters have presented the values obtained for D_o, $N\alpha E$ (chapter VI), N (chapter VII), and α (chapter VIII). Thus the value of E can now be determined as the quotient of $N\alpha E$ divided by N times α. The neoblast survival curve parameters, D_o and E, (with their standard errors) are presented in table IX-1, and the survival curve for diploid neoblasts is drawn in figure IX-1. Although mean magnitudes of E were found to decrease in the following order: diploid, tetraploid[1], triploid neoblasts, the variation was not significant.

The value of $N_{o\,\alpha\,50}$ for both diploid and triploid D. lugubris was found to be 5.714 ± 1.633. This number can also be interpreted as the proportional excess of neoblasts above the number required to yield a survival probability of 50%. Thus approximately one sixth of the neoblasts in a planarian are prerequisite to a 50% chance of survival.

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1. Assuming α is approximately the same as for triploids. N and $N\alpha E$ for the tetraploids are not significantly different from the respective values for triploids.

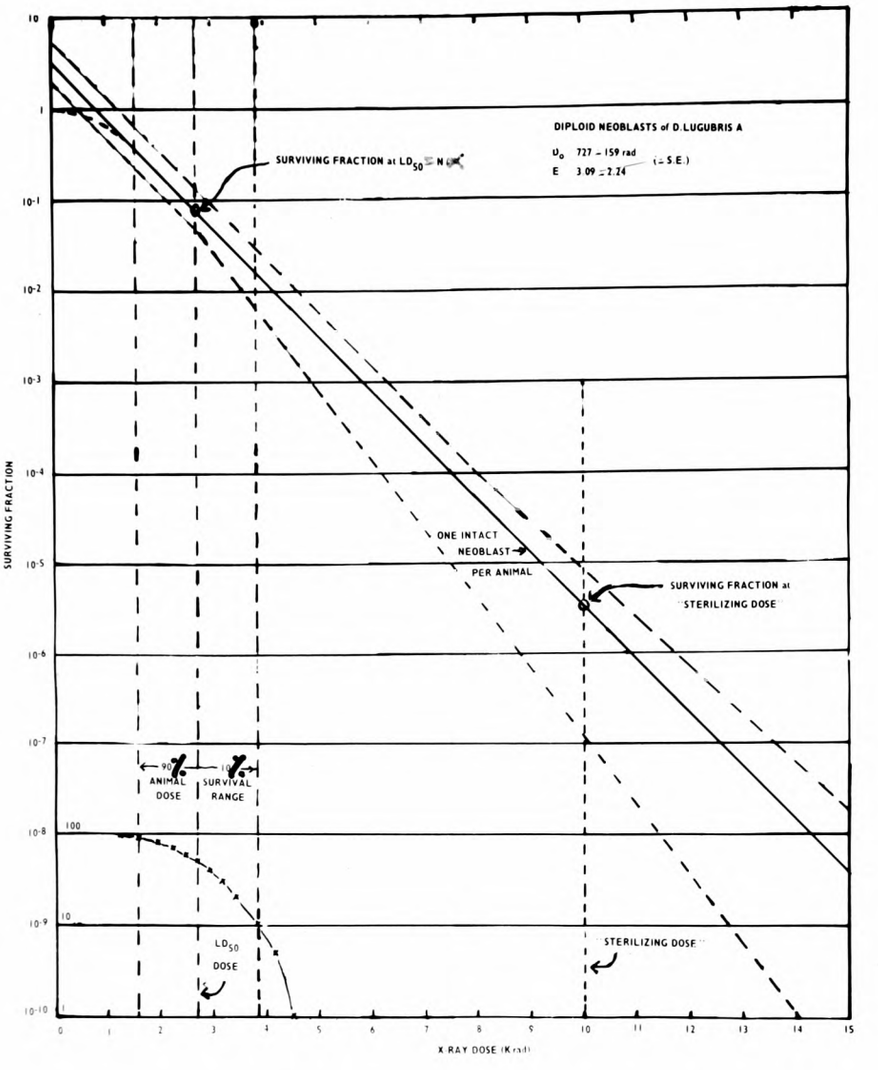

Figure IX-1

Dose response curve deduced for neoblasts of diploid D. lugubris

In the lower left corner we see a graph of % animal survival with dose. Dotted lines indicate the dose range for 90%-10% survival. The neoblast curve is extrapolated to show the dose which leaves one intact neoblast per animal, and the degree of depopulation at 10 krad. The dashed lines flanking the neoblast curve are ± one standard error of the mean.

Table IX-1

D. lugubris Neoblast Survival Curve Parameters

Biotype	Ploidy	D_o ± S.E.	E ± S.E.
A	2n	727 ± 159	3.09 ± 2.32
B	3n	780 ± 172	0.62 ± 0.35
D	4n	983 ± 279	0.67* ± 0.56

* Based on the assumption that α_{50} for the tetraploids is approximately equal to α_{50} for the diploids and triploids, i.e. $\alpha_D = 4.93 \times 10^{-5}$, the mean of the diploid and triploid α_{50}'s. 95% error brackets of α_A + 2 S.E. to α_B - 2 S.E. were assumed, yielding a S.E.(α_D) = ± 0.51 x 10^{-5}.

Chapter X

A Test of a Prediction of the Model

Chapter X

A Test of a Prediction of the Model

One of the relationships predicted by the model (q.v. chapter VI) is that

$$LD_{50} = D_o \left[\ln(N\alpha E) + 0.65\right] .$$

As we have already obtained values of N, α, D_o, and E, it should now be possible to predict the changes in LD_{50} following given changes in N. Decapitation has been shown to have no significant effect on planarian postirradiation survival (chapter V), so if α and E remain unchanged after decapitation, the LD_{50}'s of pieces of planarians (i.e. containing known numbers of neoblasts) cut postirradiation should vary in a defined and predictable manner.

The predicted LD_{50} values and their respective standard errors, for varying proportions of the initial number of neoblasts, N_o, are presented in table X-1. In order to test these predicted values, two types of experiment were performed. The first set of experiments involved 24h postirradiation cutting of planarians to produce pieces containing 16% (20% by length), 40%, 60%, and 84% (80% by length) of the planarians neoblasts. The LD_{50} for each of these size groups was then compared with the predicted value. Pieces of the same size group but differing in cephalo-caudal origin were treated as separate subgroups. The results of these experiments for D. lugubris A and B and Polycelis tenuis-hepta are

presented in tables X-2 and 3, and are compared (D. lugubris only) with the predicted values in table X-1.

The net result was that although no systematic or significant change in LD_{50} was observed with respect to size of piece or cephalo-caudal origin, neither did the experimental results differ significantly from the predicted values. However, the cutting may have influenced α, for example, in such a way as to cast doubt on the assumption that only H was being varied.

The second set of experiments altered H by shielding a variable given proportion of each animal during the radiation sterilization of the remaining portion. This method was thought to be less likely to affect α.

In order to obtain a reasonably sharp dose cut-off at the edge of the shields, $^{90}Sr/^{90}Y$ β^- rays were used.[1] About 1% of the 10 krad ablation dose penetrated the shields in the form of Bremsstrahlung, but all β^- rays were stopped. The R.B.E. of $^{90}Sr/^{90}Y$ β^- rays (with respect to 300 kVp X-rays) is reported in Appendix II. The results of these experiments with diploid and triploid D. lugubris in which 80%, 50%, or 0% of each animal was radiation sterilized, are presented in table X-4, and are compared with the predicted values in table X-1. Again, none of the observed values were signif-

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1. Details of the experimental method are given in chapter IV B.

icantly different from the expected(predicted) values. Moreover, LD_{50} decreased in the expected manner - e.g. for biotype B the decrease in LD_{50} of animals 20% of which were shielded, compared with animals 50% or 100% of which were shielded, was found to be highly significant ($P < 0.01$). Thus, within the limits of experimental error, the variation of LD_{50} with N was in agreement with that predicted by the model.

Table X-1

Biotype	$N_o \times 10^{-5}$	$\alpha \times 10^{-5}$	E	$N_a E$	% Intact*	D_o	$\ln(N_a E)$	Predicted** X-Ray LD_{50}	Observed X-Ray LD_{50}	RBE*	Predicted** β^- LD_{50}	Observed β^- LD_{50}
A	± 1.406 ± 0.267 x 10^5	± 4.76 ± 0.16 x 10^{-5}	± 3.09 ± 2.32	± 20.70 ± 15.03	100	± 727 ± 159	3.030	± 2675 ± 715	± 2674 ± 177	± 0.805 ± 0.090	3323 ± 961	± 3320 ± 176
	" x 0.84	"	"	± 17.39 ± 13.64	80	"	2.856	± 2549 ± 729	± 3083 ± 141	"	3166 ± 971	-
	" x 0.60	"	"	± 12.42 ± 10.12	60	"	2.518	± 2303 ± 715	-	"	2861 ± 943	-
	" x 0.50	"	"	± 10.35 ± 8.72	50	"	2.337	± 2172 ± 716	-	"	2698 ± 938	± 3673 ± 248
	" x 0.40	"	"	± 8.28 ± 7.35	40	"	2.114	± 2009 ± 729	-	"	2496 ± 946	-
	" x 0.16	"	"	± 3.31 ± 4.64	20	"	1.197	± 1343 ± 1034	± 2626 ± 171	"	1668 ± 1300	± 465 ± 1701
B	± 1.406 ± 0.267 x 10^5	± 5.10 ± 0.18 x 10^{-5}	± 0.624 ± 0.352	± 4.48 ± 2.39	100	± 780 ± 172	1.500	± 1677 ± 490	± 1677 ± 143	± 0.678 ± 0.080	± 2472 ± 775	± 2472 ± 200
	" x 0.84	"	"	± 3.76 ± 2.28	80	"	1.320	± 1537 ± 526	± 1903 ± 181	"	2266 ± 817	-
	" x 0.60	"	"	± 2.69 ± 1.73	60	"	0.990	± 1279 ± 531	± 1675 ± 85	"	1885 ± 844	-
	" x 0.50	"	"	± 2.24 ± 1.52	50	"	0.806	± 1136 ± 547	-	"	1674 ± 829	± 2185 ± 223
	" x 0.40	"	"	± 1.79 ± 1.31	40	"	0.582	± 961 ± 582	± 1935 ± 206	"	1417 ± 874	-
	" x 0.16	"	"	± 0.72 ± 0.93	20	"	-0.3285	± 251 ± 1012	± 1757 ± 226	"	370 ± 1497	± 717 ± 360

* % remaining after cut (X-rays), or % shielded (β^-). ** Predicted $LD_{50} = D_o \sqrt{\ln(N_a E)+0.65}$.

* RBE = $\frac{\text{X-ray dose}}{\beta^-\text{ray dose}}$, q.v. Appendix II. ** Predicted β^- LD_{50} = Predicted X-ray LD_{50} ÷ RBE.

Table X-2

Effect of Size of Planarial Fragment on Survival (D. lugubris)

Species	LD_{50} ± S.E.	K ± S.E.	C ± S.E.	σ_s	Size
D. lugubris					
A	2674 ± 177	873 ± 191	4.1 ± 4.2	18	100%
	2994 ± 173	428 ± 231	3.4 ± 2.1	11	78% A*
	3620 ± 562	1348 ± 472	0.0 ± 1.9	11	78% P*
	2531 ± 240	894 ± 275	0.0 ± 2.7	11	22% A
	2752 ± 253	1004 ± 366	4.9 ± 4.0	11	22% P
	2674 ± 177	873 ± 191	4.1 ± 4.2	18	100%
	3083 ± 141	348 ± 187	4.8 ± 1.8	25	78%
	2626 ± 171	924 ± 207	2.3 ± 2.3	25	22%
D. lugubris					
B	1677 ± 143	936 ± 206	12.0 ± 5.5	49	100%
	1862 ± 198	714 ± 208	26.7 ± 8.7	11	80% A
	2008 ± 246	941 ± 310	16.0 ± 9.3	11	80% P
	1803 ± 114	327 ± 118	3.2 ± 4.4	6	60% A
	1579 ± 112	263 ± 105	3.7 ± 6.6	6	60% P
	1875 ± 284	898 ± 374	0.0 ± 5.7	6	40% A
	2021 ± 207	648 ± 253	2.0 ± 6.2	6	40% P
	1592 ± 268	809 ± 294	15.4 ± 11.4	11	20% A
	1987 ± 376	1335 ± 567	21.7 ± 10.8	11	20% P
	1677 ± 143	936 ± 206	12.0 ± 5.5	49	100%
	1903 ± 181	896 ± 222	19.7 ± 6.7	29	80%
	1675 ± 85	294 ± 90	3.1 ± 4.2	15	60%
	1935 ± 206	833 ± 288	0.0 ± 5.6	15	40%
	1757 ± 226	1043 ± 281	18.7 ± 8.1	29	20%

* A = Anterior; P = Posterior

Table X-3

Effect of Size of Planarial Fragment on Survival (Polycelis)

Species	LD_{50}	± S.E.	K	± S.E.	C	± S.E.	J_s	Size
Polycelis tenuis-hepta	981	± 235	564	± 257	8.6	± 12.1	5	100%
	999	± 239	705	± 291	9.8	± 11.9	3	80% A*
	1249	± 130	597	± 153	0.0	± 3.2	3	80% P*
	919	± 110	506	± 130	0.0	± 3.2	3	60% A
	1121	± 141	521	± 148	0.0	± 4.1	3	60% P
	781	± 222	579	± 245	11.1	± 13.3	3	40% A
	979	± 184	930	± 325	0.0	± 3.2	3	20% P
		-		-		-	-	20% A
		-		-		-	-	20% P
	706	± 104	388	± 94	1.9	± 3.1	36	100%
	1140	± 121	661	± 143	5.0	± 5.3	9	80%
	1017	± 78	513	± 89	0.0	± 2.2	9	60%
	863	± 152	794	± 216	5.0	± 5.6	9	40%
		-		-		~ 55%	-	20%

* A = Anterior; P = Posterior

Table X-4

Effect of Shielding on Survival of D. lugubris after $^{90}Sr/^{90}Y$ β^- irradiation

Species	LD_{50}* ± S.E.	K* ± S.E.	%C ± S.E.	σ_s	% Shielded
D. lugubris A	3320 ± 176	595 ± 244	19.1 ± 7.2	27	100
	3673 ± 248	1993 ± 415	10.5 ± 3.4	27	50
	465 ± 1701	4341 ± 3211	40.0 ± 10.4	27	20
D. lugubris B	2472 ± 200	967 ± 270	1.9 ± 3.1	27	100
	2185 ± 223	1055 ± 321	11.4 ± 7.3	27	50
	717 ± 360	1064 ± 310	31.6 ± 10.9	27	20

* in units of $^{90}Sr/^{90}Y$ β^- rad in soft tissue.

Chapter XI.

Rate of Neoblast Disappearance Postirradiation

Chapter XI

Rate of Neoblast Disappearance Postirradiation

A Methods

1 Pre-fixation Methods

The animals used for this study were D. lugubris biotype B, obtained in the same manner as described in chapter VII. After four days starvation they were measured, by the method given in chapter VII, and 65 animals of 8.0 ± 0.5 mm in length were set aside for the experiments. The following day 63 of these animals were irradiated in three groups of 21 animals with 2, 5 or 10 krad of 300 kVp X-rays by the method described in chapter IV. Immediately the irradiations were finished, three animals from each group plus the two unirradiated animals were fixed and embedded, by the methods described in chapter VII (cover slip and formol-Zenker), to serve as controls. The rest of the animals were set up in groups of three in 50 ml crystal polystyrene tubes of mains water and kept at 20° C. Thereafter, on days 2, 5, 7, 10 and 15 postirradiation, a tube of three animals was selected from each dose-group and the animals fixed and embedded as were the controls.

None of the 5 or 10 krad, and only one of the 2 krad animals, set aside for fixation on day 20 postirradiation survived until that time. The 2 krad animal was not killed on

day 20 but lysed on day 25 postirradiation.

2 Post-fixation Methods

After embedding in paraffin wax (56°C m.p.), the animals were serial-sectioned at 10μ, deparaffinized, and stained in Azure B (q.v. chapter VII) for neoblast counts.

The 20% sample counts of chapter VII were extremely laborious to obtain. In view of the agreement between our estimate of the total number of neoblasts per animal based on the corrected data of Lender and Gabriel (q.v. chapter VII) and that based upon our own data, it was decided that by a judicious choice of which sections to count, sufficient accuracy (for relative comparisons) could be obtained by counting 25 sections per animal.

The sections chosen for counting were located by reference to anatomical position along the cephalo-caudal axis of the animal so that counts from different animals would be comparable. The five sections centered on each of the following anatomical positions:

(1) the level of both eyes (E)

(2) the level of the middle (by length) of the brain (B)

(3) the level three sections anterior of the anterior-most portion of the pharynx (S)

(4) the level of the middle (by length) of the pharynx (M)

(5) the level halfway (by length) between the end of the pharynx and the posterior tip of the animal (H)

were counted for each animal

B Results

Mean counts with their standard errors were found for each position on each animal and for all the animals of a given dose and postirradiation fixation-time. From table XI-1 and figure XI-1 we see that all five regions show a common pattern of neoblast loss with time, and a tendency for the posterior positions to deplete to a slightly greater extent. This latter observation might be due to forward migration as anterior necrosis sets in. The level of the eyes has fallen off (lysed) by day 15 after 10 krad. Figure XI-2 shows a similar pattern with dose. Again, the posterior regions deplete more rapidly. Between 10 and 15 days after 10 krad, the rate of depletion decreases.

An estimate of the mean total number of neoblasts per animal for each dose-time was obtained by plotting the mean number of neoblasts for each position against the coordinate of that position along the animal's cephalo-caudal axis, connecting the points with straight lines, and calculating the area under the resultant distribution curve (e.g. figure XI-2). As the animals were of uniform size and the datum of interest is the proportion, relative to the control, of neoblasts remaining at various times postirradiation (rather than the absolute number per se), systematic errors in the estimate of total neoblast cellularity due to deviations of

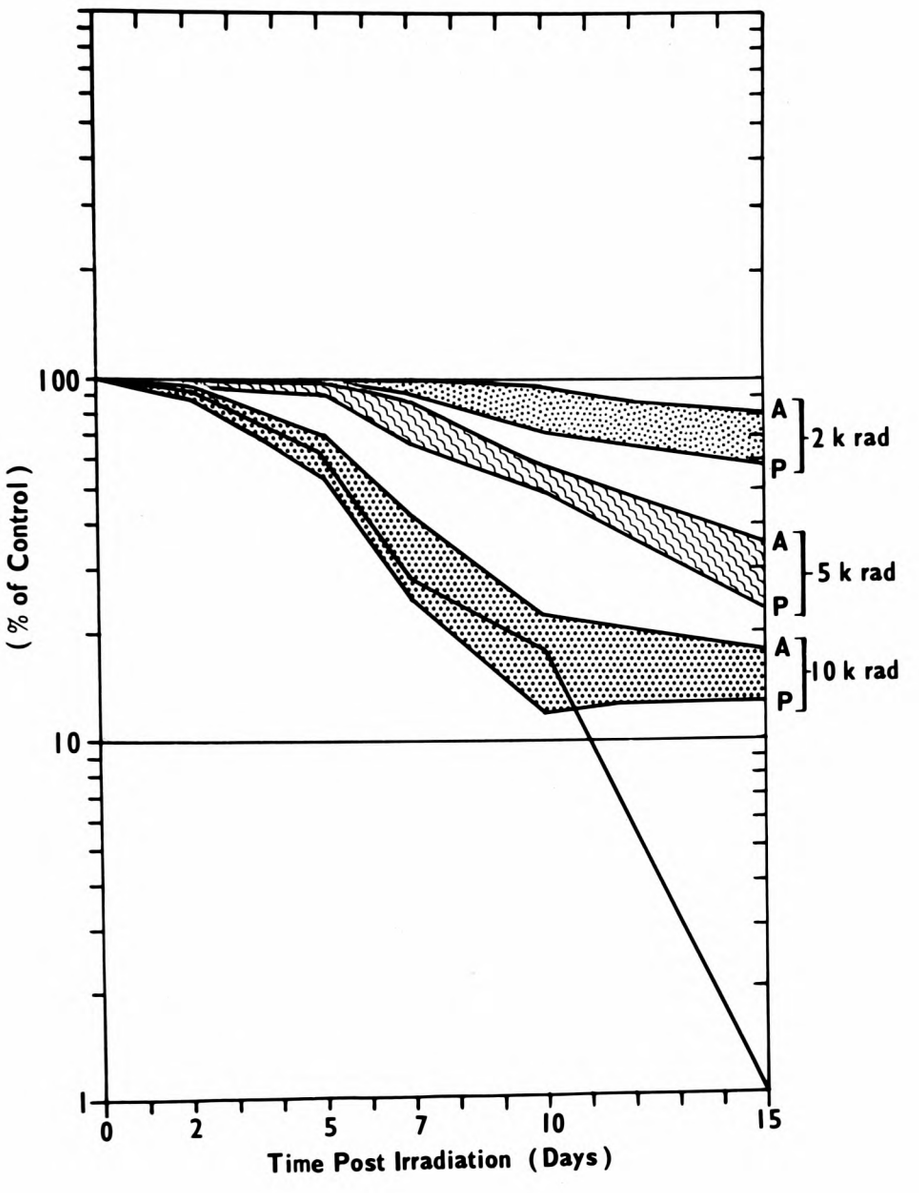

Figure XI-1

Range of rates of neoblast loss with time at various positions along the cephalo-caudal axis

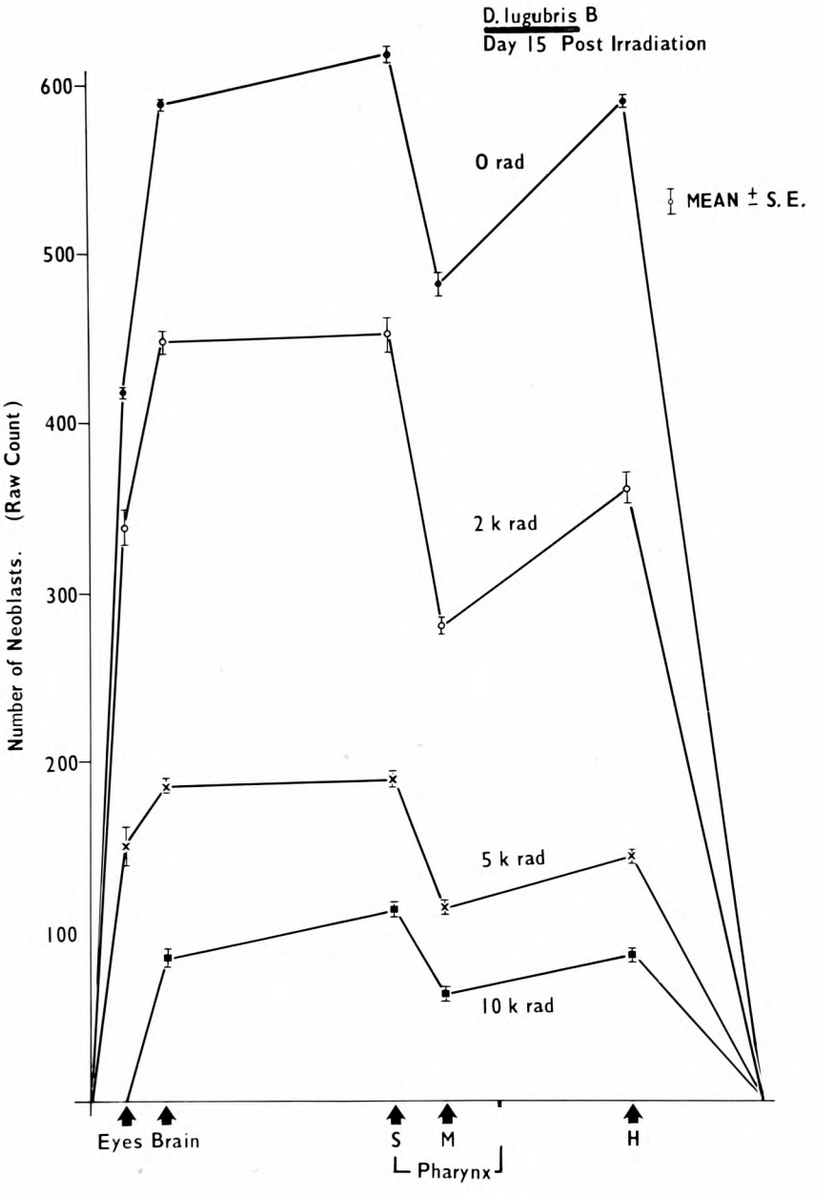

Figure XI-2

Neoblast distribution and rate of neoblast loss with dose

this curve from the true distribution curve are unlikely to be of importance (see below).

The coordinates of the five positions (E, B, S, M and H) along the planarian's long axis were, respectively, 1.0, 2,2, 9.0, 10.5, and 16.1 with the full length of the animal normalized to 20.0 units. If 1.0 units along the ordinate were taken to be equivalent to a gross count of 20 neoblasts per section, a unit of area would be equivalent to 12 cells per animal (corrected by Abercrombie's (1946) formula and assuming that all animals had shrunk to 50% of their live length, i.e. to 4.0 mm). The area under the distribution curve can be expressed as a linear function of the counts at each of the five preselected anatomical positions.

Thus,

$$A = 0.5E + 1.2\left(\frac{E+B}{2}\right) + 6.8\left(\frac{B+S}{2}\right) + 1.5\left(\frac{S+M}{2}\right) + 5.6\left(\frac{M+H}{2}\right) + \frac{3.9H}{2}$$

$$= 1.1E + 4.0B + 4.15S + 3.55M + 4.75H$$

where E, B, S, M and H represent the mean gross counts of neoblasts per section at their respective anatomical positions; and the total number of neoblasts per animal, N, = 12A.

The variance of a weighted sum is equal to the sum of the products of the squared standard deviation and the squared weight for each mean. Thus the variance of the total number of neoblasts per animal is given by the equation:

$$\mathrm{Var}(N) = 12\ \mathrm{Var}\ (A)$$

$$= 12\ \left[(1.1)^2\sigma_E^2 + (4.0)^2\sigma_B^2 + (4.15)^2\sigma_S^2 + (3.55)^2\sigma_M^2 + (4.75)^2\sigma_H^2\right]$$

and the standard error of the mean is then:

$$S.E.(N) = 12\ S.E.(A) = 12\sqrt{\frac{Var(A)}{n}}$$

$$= 12\left\{1.21\left[S.E.(E)\right]^2 + 16\left[S.E.(B)\right]^2 + 17.22\left[S.E.(S)\right]^2 + 12.6\left[S.E.(M)\right]^2 + 22.56\left[(S.E.(H)\right]^2\right\}^{\frac{1}{2}}$$

Table XI-1 and figure XI-3 show that the behaviour of the animal as a whole is much as was that of its parts. We also see that higher doses produce higher depletion rates. Figure XI-4 demonstrates the change in the shape of the dose vs. neoblast-disappearance curve with time. This suggests that the shape of histologically determined "survival curves" (i.e. per cent disappearance with dose) may well be dependent upon the choice of end-point time and thus present a distorted picture of the end result. In such cases it would be desirable to have further data to confirm that the curve does not continue to change after the chosen endpoint time. In the present case we see that the curve shifts from a concave-upwards to a linear-exponential and could possibly become convex-upwards.

Measurement of the length and volume of each animal (by the methods of chapter VII) shows a significant increase in fixed length with postirradiation time but not with dose. Volume, on the other hand, showed no significant changes with dose or time (Corner Test as in chapter VII). This may be a reflection of the decreased flattening (during fixation under the coverslip) applied to specimens at later postirradiation

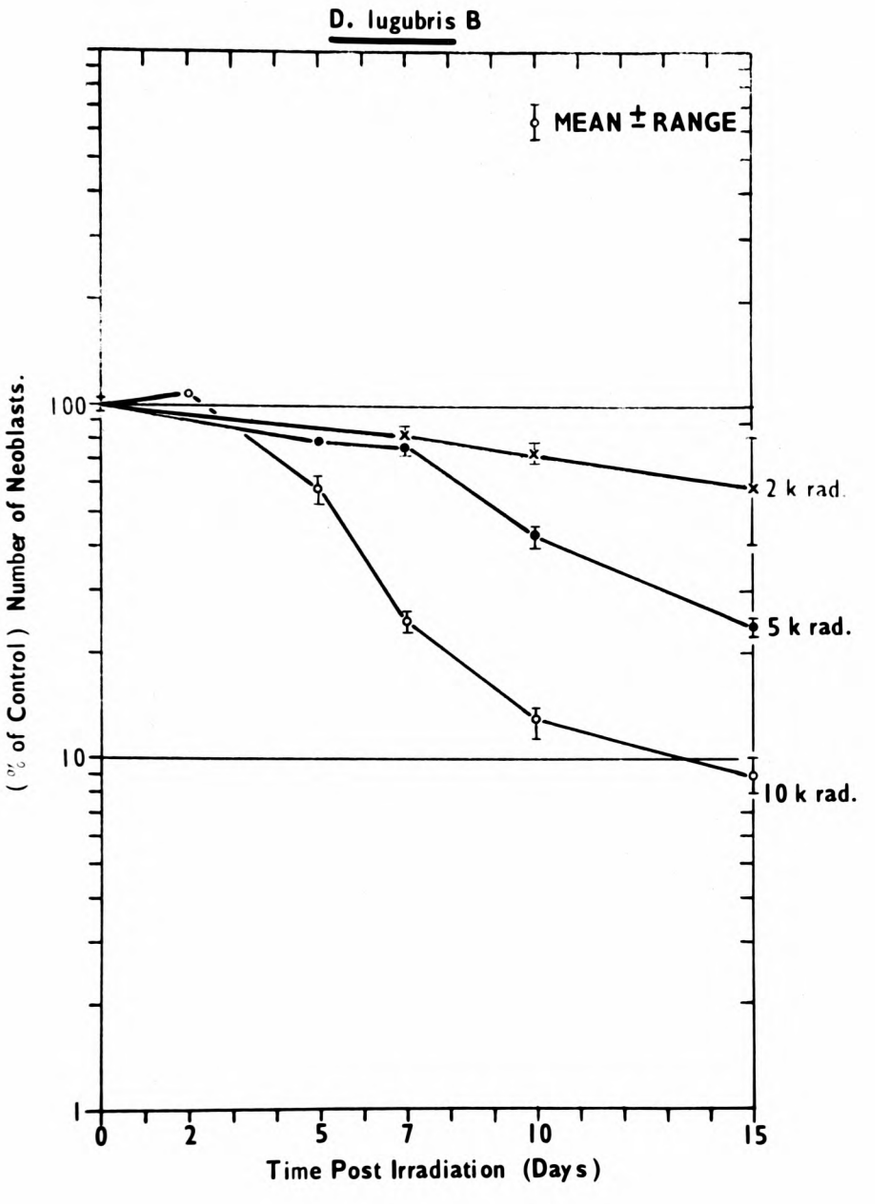

Figure XI-3

Rate of neoblast loss with time

(entire animal)

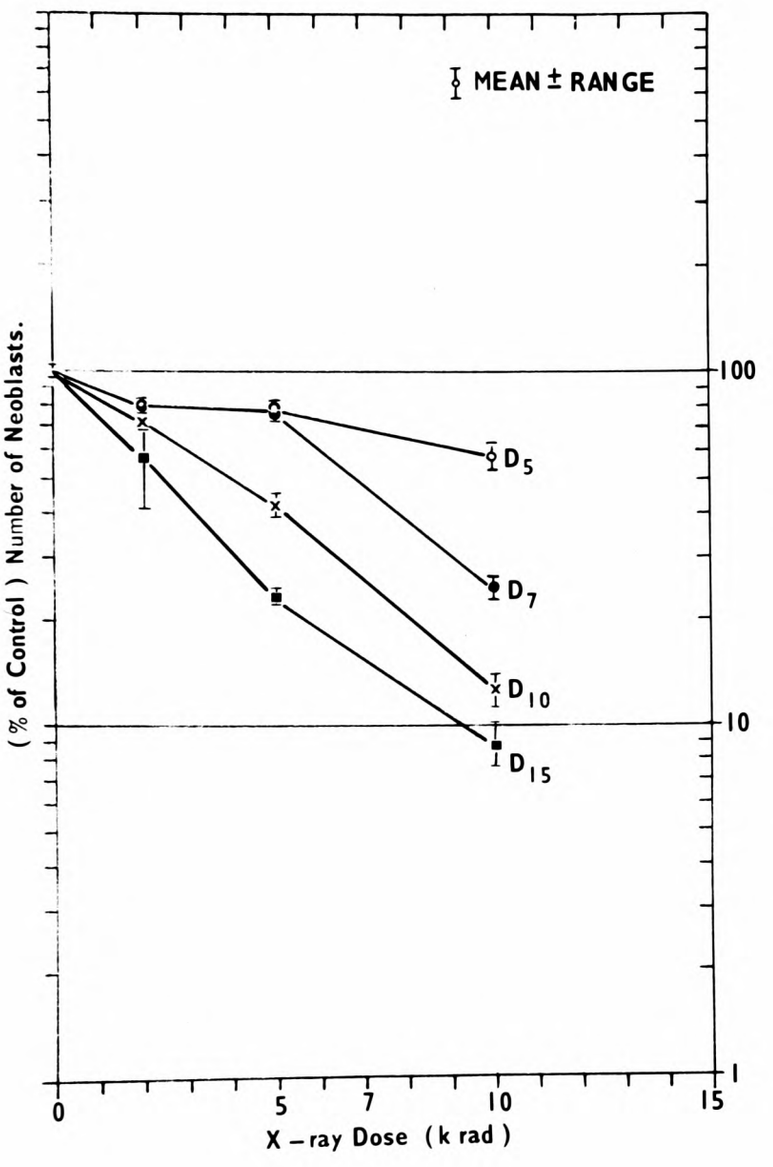

Figure XI-4

Time dependence of the dose vs neoblast disappearance curve

times due to increased specimen fragility and a fear of breaking the animal's weakened epithelium by excessive flattening. In view of this finding, the total neoblasts per animal estimates were corrected by use of the appropriate fixed length for animals fixed at each dose and postirradiation time.

It is also of interest to note that the value of α^{-1} obtained for 10-12 mm long triploid D. lugubris ($\sim 2.0 \times 10^4$, q.v. chapter VIII) is ~11.5% of the normal number of neoblasts to be found in a planarian of that size ($\sim 1.75 \times 10^5$). As this corresponds to the number of reproductively intact neoblasts necessary for a 50% probability of survival, 60 days later, it becomes obvious that there is no 1 : 1 correlation between reproductively intact and histologically intact neoblasts.

Table XI-1

Dose and Time Dependence of the Postirradiation Distribution of Neoblasts

Dose	Time	E	B	S	M	H	N	S.E.(N)
Control	0	418.5 100*	588.9 100	618.3 100	483.4 100	590.0 100	146120 100	± 554
10 krad	D_2	395.0 94.4	568.6 96.6	608.1 98.4	437.5 90.5	570.8 96.7	158810 108.7	± 441
	D_5	263.5 63.0	404.0 68.6	438.9 71.0	281.3 58.2	324.6 55.0	84390 57.8	± 551
	D_7	118.6 28.3	204.7 34.8	262.8 42.5	152.6 31.6	145.1 24.6	36009 24.6	± 396
	D_{10}	74.1 17.7	87.8 14.9	138.1 22.3	61.3 12.7	70.9 12.0	18652 12.8	± 293
	D_{15}	0 0	84.0 14.3	112.0 18.1	62.3 12.9	86.3 14.6	12926 8.8	± 316
5 krad	D_5	406.1 97.0	604.9 102.7	622.9 100.7	487.5 100.8	534.3 90.6	115765 79.2	± 348
	D_7	309.6 74.0	505.4 85.8	536.1 86.7	334.1 69.1	389.9 66.1	112655 77.1	± 421
	D_{10}	243.3 58.1	282.7 48.0	349.9 56.6	239.6 49.6	286.1 48.5	62211 42.6	± 308
	D_{15}	150.5 36.0	186.3 31.6	189.2 30.6	112.4 23.3	143.3 24.3	34074 23.3	± 357
2 krad	D_7	425.5 101.7	574.3 97.5	607.1 98.2	444.9 92.0	531.5 90.1	119302 81.6	± 405
	D_{10}	375.9 89.8	504.5 85.7	588.2 95.1	344.9 71.3	448.6 76.0	106416 72.8	± 950
	D_{15}	339.1 81.0	449.1 76.3	453.4 73.3	279.1 57.7	361.3 61.2	85669 58.6	± 894

* % of control.

Chapter XII

The Effect of Radiation on Head-Frequency

Chapter XII

The Effect of Radiation on Head-frequency

As we remember from the introduction, the experimental results of Dubois (1949) and Chandebois (1963b, 1965a, b) on the extinction of head regeneration ability, were seen by these authors to be mutually contradictory. Although these results could be reinterpreted in such a way as to remove the contradiction, similar experiments were performed by us so as to have more complete and more comparable data. The two species used for this investigation were D. lugubris B (the material of Stéphan-Dubois' experiments) and D. etrusca, a member of the 'gonocephala' group (Benazzi, 1965b, personal communication) and thus closely related to Chandebois' D. subtentaculata (= D. gonocephala, q.v. footnote 2, page 275, Benazzi, 1960b).

A Methods

Planarians were irradiated (as in chapter III) with 0, 1, 2, 3, 4, 5 or 10 krad and then, in groups of 20, were decapitated at the level of auricles three or 27 hours postirradiation. After decapitation the planarians were kept individually in 13 ml transparent crystal polystyrene tubes of mains water at 20°C and were examined daily (under a Zeiss stereoscopic dissecting microscope at x40) for wound closure,

presence of new tissue, appearance of eye pigment cups, appearance of complete eyes (the endpoint for complete head regeneration), and mortality.

B Results

In table XII-1 and figure XII-1 we see that for D. etrusca decapitated 3 hours postirradiation, even the 10 krad animals are capable of regenerating complete heads. Thus D. etrusca are quite comparable to D. subtentaculata. Regeneration occurs somewhat more rapidly in the unirradiated animals but even after 10 krad (7-8 x LD_{50}) the delay is only of the order of 50%. For the controls, no eyespots (pigment cups) appeared before day four, 45% appeared on day 4 and 100% had appeared by day 5. The one to four krad groups had their eyes regenerated by day 6 to 8 and the five and 10 krad groups reached their maxima about the same time. No further regeneration occurred and after day 13 mortality intervened.

The 27 hour postirradiation decapitated D. etrusca controls behaved much as did the 3 hour controls (q.v. table XII-2). Through days four to six, the effect of increasing dose is much more noticeable in the 27 hour than in the 3 hour group. After day 7 there is no further regeneration and resorption of the head (at 5 and 10 krad) begins. Between day 13 and 24 mortality begins to occur in the 10 krad group, spreading to lower dose groups thereafter. Thus the

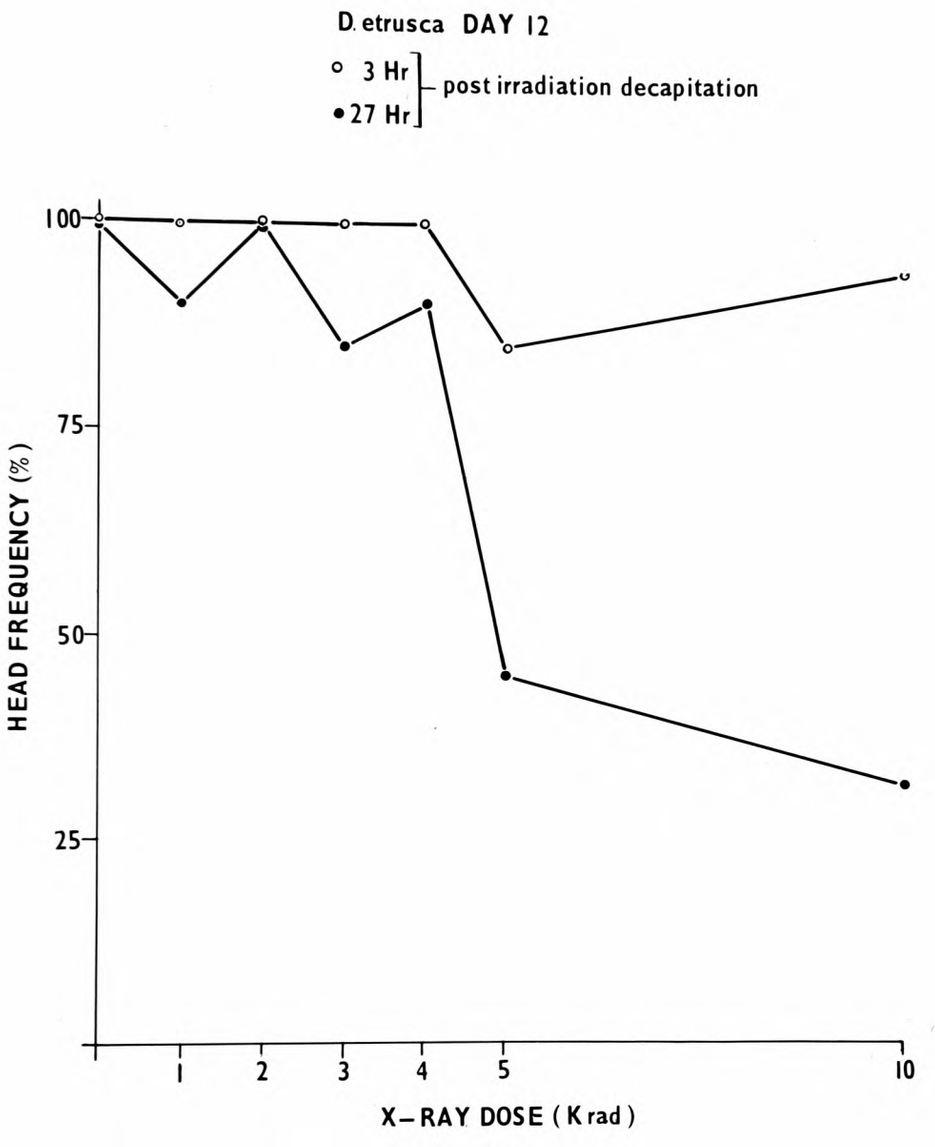

Figure XII-1

Dose vs head-frequency curve for D. etrusca

data for D. etrusca agree with the data of Chandebois for D. subtentaculata and suggest that these results could be considered to apply to other members of the 'gonocephala' group.

Similarly, tables XII-3 and XII-4 and figure XII-2 confirm the findings of Stéphan-Dubois for D. lugubris. We also note that for the 3 hour postirradiation decapitated group, the loss of head-frequency is almost linear with dose ($\sim$-10.6% per krad) above LD_{50} doses. For animals decapitated 27 hours postsupralethal-irradiation, almost no regeneration is possible. Even at 4 and 5 krad, the few animals which were able to produce eyes resorbed them again a day or two later. However, for doses of 3 krad and below, head-frequency remains almost unaltered.

Thus the results of both authors are confirmed. D. lugubris, although much more radioresistant than D. etrusca (with respect to LD_{50} for animals and $D_{\bullet}$ for neoblasts) is, paradoxically, much more readily inhibited from head regeneration by supralethal doses of irradiation. Moreover, the latent headforming ability decays much more rapidly with postirradiation time in D. lugubris than in D. etrusca.

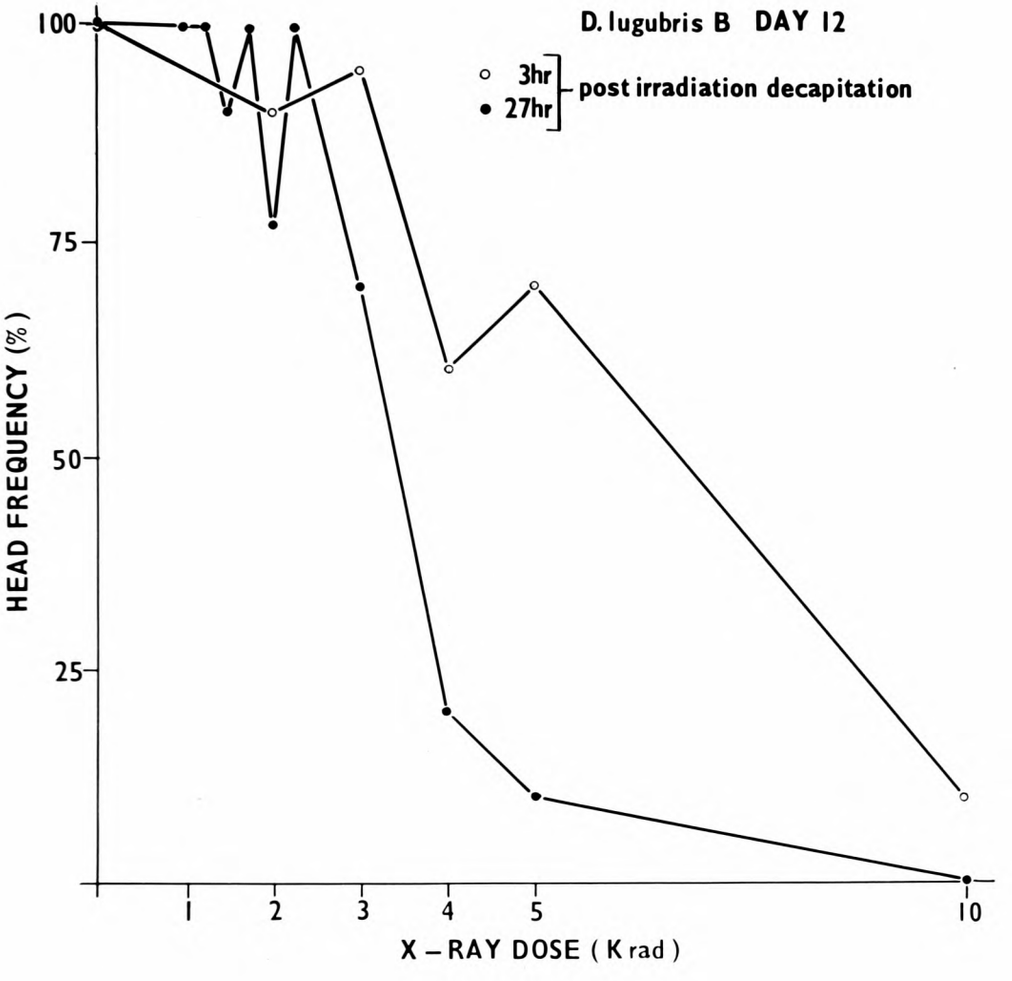

Figure XII-2

Dose vs head-frequency curve for D. lugubris B

Table XII-1

Effect of 3 Hour Postirradiation Decapitation on X-ray Dose-dependence of Head-frequency and Mortality Probability in D. etrusca

t post decap. (days)	Dose (krad) 0	1	2	3	4	5	10
4	45*	20	5	20	20	20	11.1
5	100	85	60	75	70	60	55.5
6	100	90	85	95	100	75 5**	83.4
7	100	90	100	95	100	80 5	94.5
8	100	100	100	100	100	85 5	94.5
12	100	100	100	100	100	85 5	94.5
13	100	100	100	100	100	85 5	94.5 5.6
15	100	100	100	100	100	5	16.6
18	100	100	100	100	100	15	33.7
19	100	100	100	100	100	25	33.7
20	100	100	100	100	10	35	50
21	100	100	100	10	10	45	61.2
22	100	100	100	15	25	45	61.2
25	100	100	30	45	65	70	77.8
26	100	100	45	60	75	80	94.4
27	100	100	45	60	90	85	94.4
29	100	100	75	70	95	100	100
60	0	0	100	100	100	100	100

* These numbers represent the head-frequency.

** These numbers represent % mortality.

Table XII-2

Effect of 27 Hour Postirradiation Decapitation on X-ray Dose-dependence of Head-frequency and Mortality Probability in D. etrusca

t post decap. (days)	Dose (krad) 0	1	2	3	4	5	10	
3	0*	0	0	0	0	0	0	
4	55	45	30	30	25	10	0	
5	95	80	65	55	35	15	22.8	
6	100	85	85	65	50	35	27.3	
7	100	90	100	85	90	45	31.8	← start of regression
12	100	90	100	85	90	45	31.8	
13	"	"	"	"	"	"	4.5**	
14	"	"	"	"	"	"	9	
24	"	"	"	"	10	5	27.3	
25	"	"	"	15	15	15	41	
26	"	"	"	30	25	15	50	
28	"	"	15	55	40	55	82	
60	0	0	90	100	100	100	100	

* These numbers represent the head-frequency.

** These numbers represent % mortality.

Table XII-3

Effect of 3 Hour Postirradiation Decapitation on X-ray Dose-dependence of Head-frequency and Mortality Probability in D. lugubris B

t post decap. (days)	Dose (krad) 0	2	3	4	5	10	
1	-	-	-	-	-	-	
2	0*	0	0	0	0	0	
5	50	15	15	20	10	0	
6	80	40	30	45	40	10	
7	90	65	50	50	55	10	
8	95	80	70	50	55	10	
9	95	80	75	50	55	10	
10	100	90	95	60	70	10	← resorption begins
12	"	5**	"	"	"	15	
13	"	5	"	"	"	20	
15	"	5	"	10	15	45	
16	"	15	5	15	35	65	
18	"	15	10	30	50	80	
21	"	30	20	55	75	85	
22	"	30	20	55	80	90	
23	"	35	20	75	80	90	
25	"	35	35	80	90	100	
60	0	58	40	100	100	100	

* These numbers represent the head-frequency.

** These numbers represent % mortality.

Table XII-4

Effect of 27 Hour Postirradiation Decapitation on X-ray Dose-dependence of Head-frequency and Mortality Probability in D. lugubris B

t post decap. (days)	Dose (krad) 0	2	3	4	5	10	
1	0*	0	0	0	0	0	
4	0	0	0	0	0	0	
5	20	0	0	0	0	0	
6	60	0	0	0	0	0	
7	75	20	10	10	5	0	
8	90	20	10	10	5	0	← resorption begins
9	100	45	55	15	10	0	
					5**		
11					20		
12					35		
14				5	45	30	
15	100	75	70	20	10	0	
		5		20	55	45	
16		10		25	70	45	
19		25	20	45	85	80	
20		30	30	70	95	85	
21		30	30	75	95	90	
23		35	30	85	95	95	
60	0	60	45	100	100	100	

* These numbers represent the head-frequency.

** These numbers represent % mortality.

Chapter XIII

The Effect of Radiation on Spontaneous Fission

Chapter XIII

The Effect of Radiation on Spontaneous Fission

As we noted in the introduction, Chandebois (1963b) stated that irradiated D. subtentaculata undergo spontaneous fission as actively as do the controls, but never regenerate. However, as Chandebois has not presented any quantitative data to support this assertion, the following analysis of our data for D. etrusca (a closely related species) was done to test the generality of this assertion.

In eight dose-survival experiments for D. etrusca, observations were also made on the number of fissions each animal underwent and the fate of the resultant pieces. The qualitative picture confirms the report of Chandebois, i.e. even after supralethal doses, fission occurs but regeneration is seldom completed. Quantitatively, however, the picture is more interesting. The total number of fissions (= total number of new individuals produced) was counted for each dose in each experiment and expressed as the proportional increase in population had all pieces survived. Thus if 10 fissions had occurred among the 21 animals set up at a given dose, then the proportional increase was 10/21 = 47.6%. By graphing these data against X-ray dose, and application of the "Corner Test for Association" (Olmstead and Tukey, 1947) a significant

correlation was found ($0.02 < P < 0.05$). Thus the number of fissions decreases significantly with increasing dose.

The above finding led to the question - is the process of fission per se inhibited by the irradiation? In an attempt to answer this question, the above data were treated as the resultant of two component contributions, i.e. fissions in animals which were to survive the doses given and fissions in animals which were to die from the irradiation. Such a resolution of the data, treated by the above methods, shows the absence of significant correlation between fissions and dose for both survivors ($P > 0.1$) and mortalities ($P > 0.05$). However, the median X-ray doses and % fissions for the mortality and survivor groups (1600 rads, 25.5%; 1000 rads, 50%; respectively) show the reason for the correlation found when all animals are considered together. Thus the decrease in the number of fissions with increasing X-ray dose is due to the death of the mortality group and its consequent cessation of fission.

Chapter XIV

General Discussion

Chapter XIV

General Discussion

The aim of this thesis has been to throw some further light on the mechanism of the radiation effect at the cellular level. We have examined the role of the cell in the organism, in an attempt to understand the survival of the organism in terms of the reproductive integrity of its constituent cells. We have also studied the role of the chromosome complement of the cell, in an attempt to relate survival to degree of polyploidy.

Our study of the literature has shown that the planarian is an organism consisting of two cell populations: non-replicating differentiated tissues interspersed with a population of morphologically identifiable stem cells. As it became obvious that the planarian could not be considered as a simple culture chamber for stem cells, the factors which link planarian survival to the reproductive integrity of its stem cells (neoblasts) were examined. This led to the construction of a simple model quantitatively relating planarian mortality probability to the parameters of the neoblast survival curve.

Examination of the mean time of death and the level of postirradiation survival (% survivors or LD_{50} and K) under several pre- and postirradiation conditions known to influence planarian regenerative ability revealed the following pattern.

(1) Variation of preirradiation metabolic rate has no effect on time of death or on % survivors.

(2) Decreased postirradiation metabolic rate postpones the time of death in all species but increases % survivors only in the fissiparous species, D. etrusca.

(3) Stem cell mobilization by pre- and postirradiation decapitation has little or no effect on % survivors, and the time of death is also unchanged (except for postirradiation mobilized Polycelis whose survival time is lengthened, and preirradiation mobilized D. etrusca whose survival time is shortened).

(4) Age, size and sexual maturity have no effect on planarian postirradiation survival.

(5) Antibiotics have no effect on % survivors but increase survival time in both sexual and fissiparous species. This may be due to a possible antibiotic-induced decrease in the planarial metabolic rate.

The difference in response observed following treatments altering metabolic rate for sexual vs. fissiparous planarians need not imply different metabolic patterns. The same result might be expected to follow from a quantitative difference of sufficient magnitude to yield a qualitatively different result. Thus the fissiparous planarian (D. etrusca)

may have a higher basal metabolic rate[1] (for given conditions of temperature and nutrition) than the non-fissiparous species (D. lugubris). Thus the starvation-induced decrease which increased survival time by 56% in D. etrusca also increased % survivors. In contradistinction to this, the lower rate for D. lugubris resulted in a 22% increase in observed survival time. This was insufficient to have a beneficial effect on % survivors.

Postirradiation stem cell mobilization had little or no effect on survival time or on % survivors. This observation, coupled with the finding that sterile neoblasts can differentiate to form normal planarian organs, suggests that maintainance of a low metabolic rate allowing a longer time for the gradual restoration of the reproductive capacity of the stem cell population is of importance to survival. The wave of stem cell mitotic and differentiative activity coupled with an increased planarial metabolic rate resulting from decapitation may be detrimental to stem cell reproductive integrity as well as to organismal survival.

Preirradiation stem cell mobilization had no effect on % survivors but, for D. etrusca, did appreciably shorten survival time. This may be a metabolic rate effect rather than an effect on neoblasts in cell cycle, and thus would be seen

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1. This is also suggested by the relative mobilities of D. etrusca and D. lugubris, the former seldom stationary, the latter seldom active.

in D. etrusca but not in D. lugubris.

Thus under conditions of low metabolic rate, where the planarian neoblasts are able to repopulate, the assumption that organismal postirradiation death is due to loss of reproductively intact stem cells seems valid.

Our model successfully defines planarian mortality in terms of the five parameters, D, N, α, D_o, and E, the first three of which have been determined independently of the planarian X-ray survival experiments, while the latter two have been deduced from the former plus the parameters of the planarian survival curve. In view of this success it is unlikely that, under our standard experimental conditions, other factors are of great importance to planarian survival. On the other hand, no quantitative model yet exists relating the survival time to metabolic rate and/or other physiological relationships. Until such a model has been formulated, it is unlikely that organismal mortality probability will be correctly predicted under all possible metabolic conditions. Even under the standardized metabolic conditions described above, this model represents the first successful quantitative description of animal mortality probability in terms of cellular reproductive integrity. Unfortunately, direct methods for the study of the neoblast X-ray dose response curve proved infeasible. Should future technical achievements change this

situation, we may be able to determine the effects of metabolic and physiological changes on N and α.

It is interesting to note that the value of $N_o\alpha_{50}$ obtained for D. lugubris suggests that this species does not have a great excess of neoblasts above that required to ensure survival. In the literature it has been generally assumed that the planarian has a superabundance of these cells.

The decreasing parenchymal density of neoblasts associated with increases in size, age and senescence suggests that neoblast-tissue density may also have to be considered in any attempt to determine postirradiation survival time.

Notwithstanding the above limitations, this study has provided a complete determination of the dose response curve of animal cells under conditions of normal growth regulation in an organized tissue in vivo. Furthermore, by this determination we have confirmed that the use of the multitarget type survival curve can be extended to give meaningful results for cells under normal in vivo conditions, where cells are not in a state of continuous turnover.

To put these results in perspective, let us examine the other cell systems studied in vivo. The first confirmation that survival curves comparable to those found by Puck

and Marcus (1956) *in vitro* also obtain *in vivo* was found by Hewitt and Wilson (1959) using a mouse leukaemia. The normal conditions of growth for this cell type, as well as the conditions under which reproductive integrity was assayed, are those of continuous turnover (exponential growth). This system, however, is analogous to the situation *in vitro* as the host has little or no effect on the growth of the leukaemic cell other than that of a culture chamber filled with nutrient material in an incubator.

Hall et al. (1962) presented the first survival curve determination for cells of an organized tissue *in vivo*. The material studied was the *Vicia* meristem where the normal conditions of growth are those of continuous turnover (exponential growth).

The haematopoietic colony forming cells, first studied by Till and McCulloch (1961) have been shown by these authors to have survival curves comparable to those of other mammalian cells *in vitro*. These cells are to be found in mammalian bone marrow and spleen and are presumably part of an organized tissue, but little is known of their normal growth conditions. The assay system in which their reproductive integrity is measured is again one of conditions of continuous turnover. Also, it is not yet clear what role these cells play in the postirradiation survival of the animal.

Hornsey (1967) has measured the values of $D_q = LD_{50f} - LD_{50s}$ after dose fractionation experiments to determine the product $D_o \ln E$ for the stem cells of the gut ($LD_{50/4}$) and bone marrow ($LD_{50/30}$) in mice. However, until the role of infection on the postirradiation syndrome can be eliminated or quantitatively defined, the validity of the assumption that LD_{50} is a directly proportional measure of stem cell reproductive integrity is very much in doubt. The findings of Byron and Lajtha (1966) indicate that haematopoietic stem cell reproductive integrity has little to do with $LD_{50/30}$ if infective commensal organisms are not controlled. The delayed gut death in germ-free mice (Wilson, 1963) may also be a reflection of the absence of infective organisms.

The studies of Withers (1967) and later of Emery (1967), do provide direct evidence of the slope of the survival curve for mouse dermis cells, an organized tissue <u>in vivo</u> but due to the limits of accuracy of the data, and the fact that the data are obtained in regions of heavy depopulation (several decades down the survival curve), extrapolation errors become very large. There is also an appreciable uncertainty as to the size of the cell population at risk. The findings of Kember (1967) for rat growth cartilage are subject to the same limitations as those of mouse dermis, but to an even

greater degree. The short range over which survival or reproductive integrity is measurable (only 1-2 decades) makes the value of extrapolation even more dubious, and the lack of a sharp endpoint makes the data highly variable.

The planarian neoblast, we have seen, is normally a resting cell, responding to the needs of the differentiated tissues as they arise. The results of the split-dose experiments with D. etrusca suggest that some neoblasts may go into cell cycle in response to a dose of radiation. However, the rate of repopulation (decay of residual injury) is such that it is unlikely that all or even most neoblasts are set into cycle. Thus our assay conditions may be very different from those of all of the above systems,[1] (except that of Hornsey) for the endpoint in those systems is clone-formation rather than maintainance of tissue function and survival. It is for this endpoint that the meaningful results we obtained with the assumption of multitarget type survival are of particular interest. It is in this sense that our survival curve determination is the first for animal cells under conditions of normal, nonexponential, growth regulation in an organized tissue in vivo.

The main sources of error in the estimates of D_o and E lie in the uncertainty ($\sim \pm$ 20%)[2] of the slope (N) determinations, and in the accumulated errors of N, α and LD_{50}.

1. At this point one might speculate that the 125-225 rad D_o for neoblasts in rapid turnover (fissiparous species) is similar to those obtained for mammalian cells, whereas the neoblasts with slow turnover rates (D. lugubris) have much higher D_o's.

2. For D_o, D. subrufa; for the fissiparous species D. etrusca and D. [illegible], this drops to $\sim \pm$ 10%.

The error of the transformation from the normalized curve predicted by the model to a probit line is small compared to the uncertainty of the slope (K) estimates and the accumulated errors, and in any case, represents a systematic error rather than a random one.

Hall's (1962) method for the determination of the parameters of survival curves of the form

$$S = 1 - (1-e^{-D/Do})^E$$

by matching a single dose to two equal fractionated doses which produce the same biological effect, is of limited use on material in a state of non-continuous turnover, such as the planarian neoblast, for the following reasons. Firstly, a necessary condition for a unique solution, is that one of the dose fractions must be small enough to fall on the initial shoulder of the dose-response curve. In the case of the planarian D. etrusca (chapter V), the repair of sublethal damage induced by doses of the order likely to fall on the shoulder is unlikely to be detectable so that only the product $D_o \ln E = (2D_f - D_s)$ could be determined. Furthermore this would require not single matched doses, but a whole series of animal survival dose response curves matched for equal conditioning dose (D_c) and $\frac{1}{2}LD_{50f}$ (i.e. for $2D_c = LD_{50f} = 2D_f$) for comparison with the single acute dose LD_{50s} determination. Secondly, a necessary condition for the use of Hall's method

is that the fraction interval must be sufficient to allow complete recovery of sublethal damage. In his system where cell turnover is exponential and continuous, the contribution of repopulation to the repair of D_c is matched by the growth of the control, in contrast to the situation for most organized tissues *in vivo* (non-continuous turnover). Thus systems of the latter type require the solution of the problem, how much of the repair of D_c is repair of sublethal damage and how much is population increase. The results for *D. etrusca* suggest that complete separation of these two components may be a serious problem. Thirdly, it is highly likely that such a population's increase between dose fractions in the split-dose group means that the cells of the two populations to be matched are not in comparable metabolic states. Thus Hall's method is best used on tissues in which the cells are in a state of constant turnover.

As a matter of self-consistency, it is interesting to see that the neoblast dose response curve for diploid *D. lugubris* gives further confirmation to what was previously assumed - that an X-ray dose of 10 krad sterilizes all of the neoblasts in a planarian (i.e. has a reasonable probability of not leaving any reproductively intact neoblasts in a given animal).

The effect of radiation on head regeneration ability suggests that some planarians possess sufficient neoblasts

to regenerate a head from radiation sterilized neoblasts (chapter XII). The time rate of postirradiation neoblast disappearance does not increase sufficiently rapidly with dose to explain the head-frequency drop from 3h to 27h post-irradiation. Therefore it seems likely that the time rate of decay of differentiation ability in the radiation-sterilized neoblast is rapid - probably a matter of hours.

In view of the errors likely to be inherent in the parameters for the conversion of the normalized mortality probability curve to its "equivalent" probit line, excessive weight should not be placed on the accuracy of the absolute values of the neoblast dose response curve. However, relative changes in D_o and E are not much affected by such errors. Thus the relative values of the extrapolation numbers are worthy of consideration. In this connection it is interesting that an Elkind-like recovery was found after fractionated doses of irradiation in D. etrusca (chapter V) which suggests that these experiments should also be carried out on D. lugubris - (1) to confirm the prediction of an extrapolation number greater than unity for the diploids and (2) as a test of the meaning of the less than unity extrapolation numbers found for the triploids and tetraploids.

Let us consider other possible meanings of, or causes for, the less than unity values of E found for the triploids and tetraploids. It might be suggested that the less than

unity extrapolation numbers indicate a non-reproductively intact portion of the population of cells identified morphologically as neoblasts. However, the hypothesised existence of such a population does not in fact explain these extrapolation numbers as the product $I\alpha_{50}$ is independent of the relative size of such a subpopulation and it is this product as the divisor of the term $\exp\left[(LD_{50}/D_o) - 0.65\right]$ which defines the extrapolation number.

Another interpretation, which would explain the low extrapolation numbers for the triploids (and possibly for the tetraploids) lies in the observation that although the animal survival curve parameters were not significantly changed by the presence of kanamycin in the postirradiation milieu (10μg/ml), the inverse slope obtained with kanamycin was lower. This would be sufficient to yield an extrapolation number of the order of four (i.e. infection might have increased the inverse slope and lowered the value obtained for E). Should this interpretation be correct, the estimates of D_o given in chapter VI would be overestimates by a factor of approximately 1.8. A redetermination of the population standards for D. lugubris with kanamycin in the postirradiation milieu might settle this question.

This thesis has been, in part, concerned with determining the importance of the multiplicity of the haploid karyogram (degree of polyploidy) as a factor in determining relative

radiosensitivity.

Our analysis of the literature has shown that of the experiments performed in attempts to solve this problem, most of the work has been done with aneuploid or embryonic material. The results of such studies would seem to be irrelevant to this question because of the effects of polysomy in the first case, and because in the second case, normal development is known to depend on more than the reproductive integrity of its constituent cells. Thus the only work of direct relevance has been that of Mortimer (1958) on haploid to hexaploid Saccharomyces, Sparrow et al. (q.v. chapter I) on diploid to dicosaploid Chrysanthemum and heptaploid to heptadecaploid Sedum. The results of Mortimer and of Sparrow et al. are in contradiction as to the protective effect of polyploidy. Sparrow et al. find a protective effect no greater than that to be expected between two different species, one with twice the chromosome number (not complement) of the other, whereas Mortimer finds a sensitizing effect for ploidy increases above diploid. Although the biological endpoint (dose rate to produce severe growth inhibition) chosen by Sparrow et al. is not a direct measure of cellular reproductive integrity, it is directly proportional to the acute dose necessary to produce lethality. Hence the relative sensitivities which they observed are unlikely to be seriously influenced by dynamic factors such as cell cycle times, and

may reflect changes in the reproductive integrity of the meristematic cells.

Thus the hypothesis that redundancy of genetic information protects by providing more "targets" is not supported by the relevant work in plants.

Although Bedford and Hall (1967) studied aneuploid (2n+1) cells of the Chinese hamster (Cricetulus) in vitro, their work is of interest because the 4n+2 line contained an exact doubling of the 2n+1 chromosomes of the hyperdiploid line, and no other data are available for animal material. Their data support the above protection hypothesis.

For the planarian, Dugesia lugubris, the values found for the parameters of the animal survival curves show that the triploid is significantly more radiosensitive than the diploid. No protective effect of polyploidy was found for the neoblasts. The parameters of the diploid neoblast survival curves are not significantly different from those of the triploid and tetraploid, but none-the-less suggest that polyploids are more radiosensitive than diploids. This finding for D. lugubris is in general agreement with the findings of Mortimer (1958) for Saccharomyces cerevisiae and does not agree with the relative radiosensitivites reported by Sparrow and colleagues (q.v. chapter I) for Chrysanthemum and Sedum or by Bedford and Hall (1967) for Chinese hamster cells

in vitro. Thus we must conclude that for the planarian, and possibly for other species at both the level of the organism and at the cellular level, there are factors of greater importance in the determination of radiosensitivity than polyploidy.

Appendix I

Oxygen Effect in Planarians

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Oxygen Effect in Planarians

The effect of irradiation under anoxic conditions was investigated in two species of planarians - namely, Polycelis felina and D. lugubris B. These studies were made in an attempt to answer the following two questions:

(1) Whether or not planarian radiosensitivity is subject to an oxygen effect, and if so, what is the magnitude of this effect in the Planariidae?

(2) Whether or not the standard conditions of irradiation (q.v. chapter IV) used in all of the X-ray experiments reported in this thesis are conditions of hypoxia.

Materials and Methods

The jig shown in figure AI-1 was made entirely of nylon. It consists of a 5 mm thick nylon 61 annulus of 1.5 inches internal diameter, cut from a 2 inch diameter rod trimmed in such a way that apart from the points of attachment of the three legs, handle, and closing screw, the walls of the annulus are 3/32 of an inch thick. The legs and handle, with adjustable nylon levelling screws, allow the jig to be accurately positioned ($\pm$ 0.5 mm) in the gas-tight pyrex glass irradiation vessel. The top and bottom of the annulus were covered with nylon bolting cloth of 90μ pore size and 35% pore

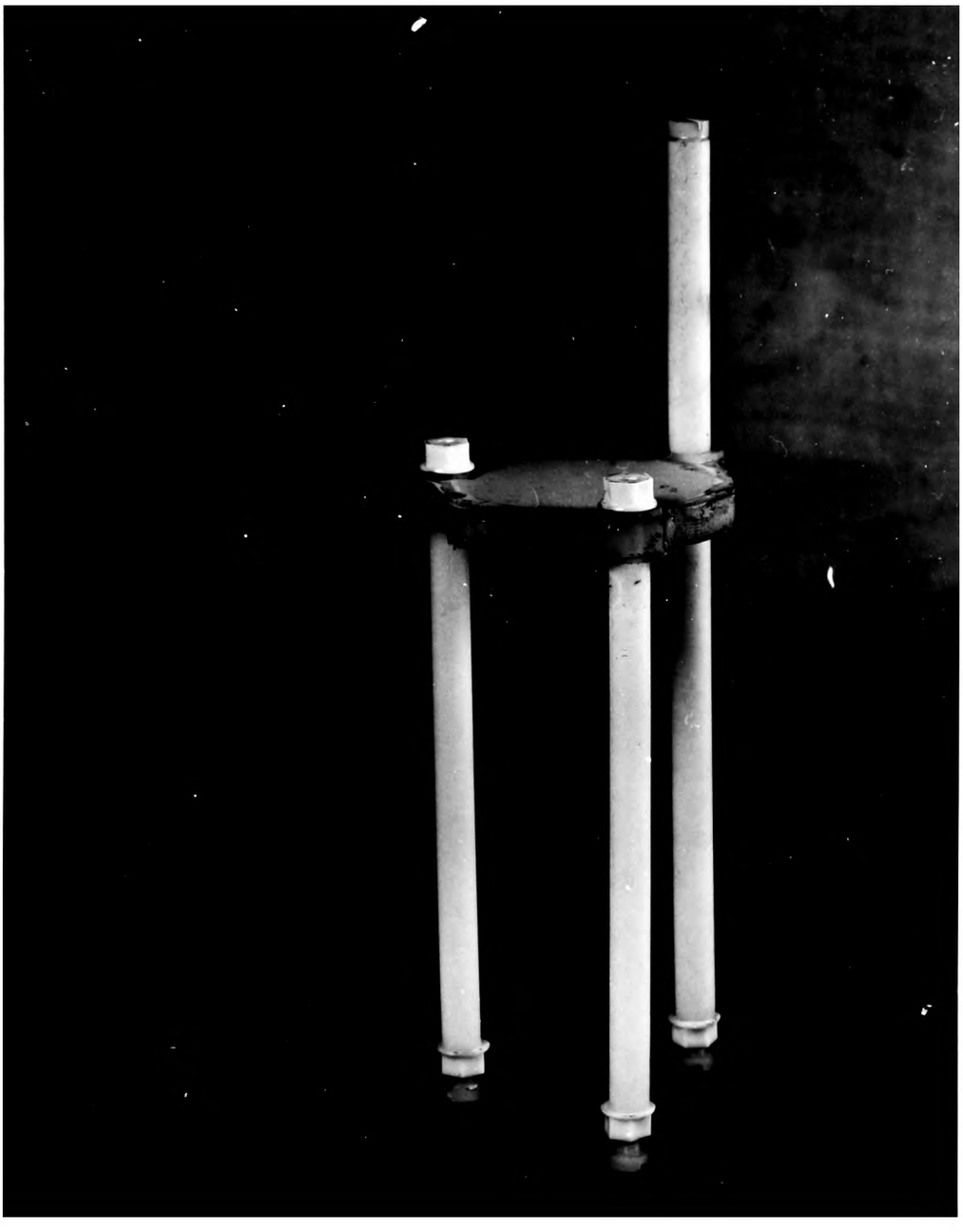

Figure AI-1

Nylon jig used for holding planarians during gassing experiments

cross sectional area[1]. The bolting cloth was fused to the annulus with a 20% (w/v) solution of nylon in hot phenol.

After pippetting animals (up to 30 per jig) into the jig, it was sealed with a flush-fitting 2BA nylon screw. The jig was then lowered into the tap-water-filled irradiation vessel until all three legs came to rest on the scintered glass filter acting as the bottom of the liquid phase portion of the vessel. Once the jig was in place, the top of the vessel was sealed with a ground glass plate and vacuum grease. As an additional precaution, bakelite clamps were used to hold the glass plate in place. A photograph of the jig in the irradiation vessel is presented in figure AI-2. Rapid exchange of water in the volume to which the planarians were confined and the rest of the water in the vessel was confirmed by several dye circulation tests.

White spot nitrogen (British Oxygen Co., $<10ppm10^6 O_2$) was introduced at the bottom of the vessel through the inlet valve (nylon 7 tubing[2] was used to connect the cylinder to the irradiation vessel), passed up through the filter in myriad fine streams of bubbles, around the jig, into the air space at the top, out through the outlet valve, through a short length of PVC tubing[2], into copper tubing, past a

1. 14N "St. Martins" Nylon, Henry Simon Ltd., Stockport, England. Equally good results were obtained using 21N "St. Martins" Nylon having a 68μ pore size and 21% of its cross sectional area open.
2. Portex, Portland Plastics Ltd., Hythe, Kent, England.

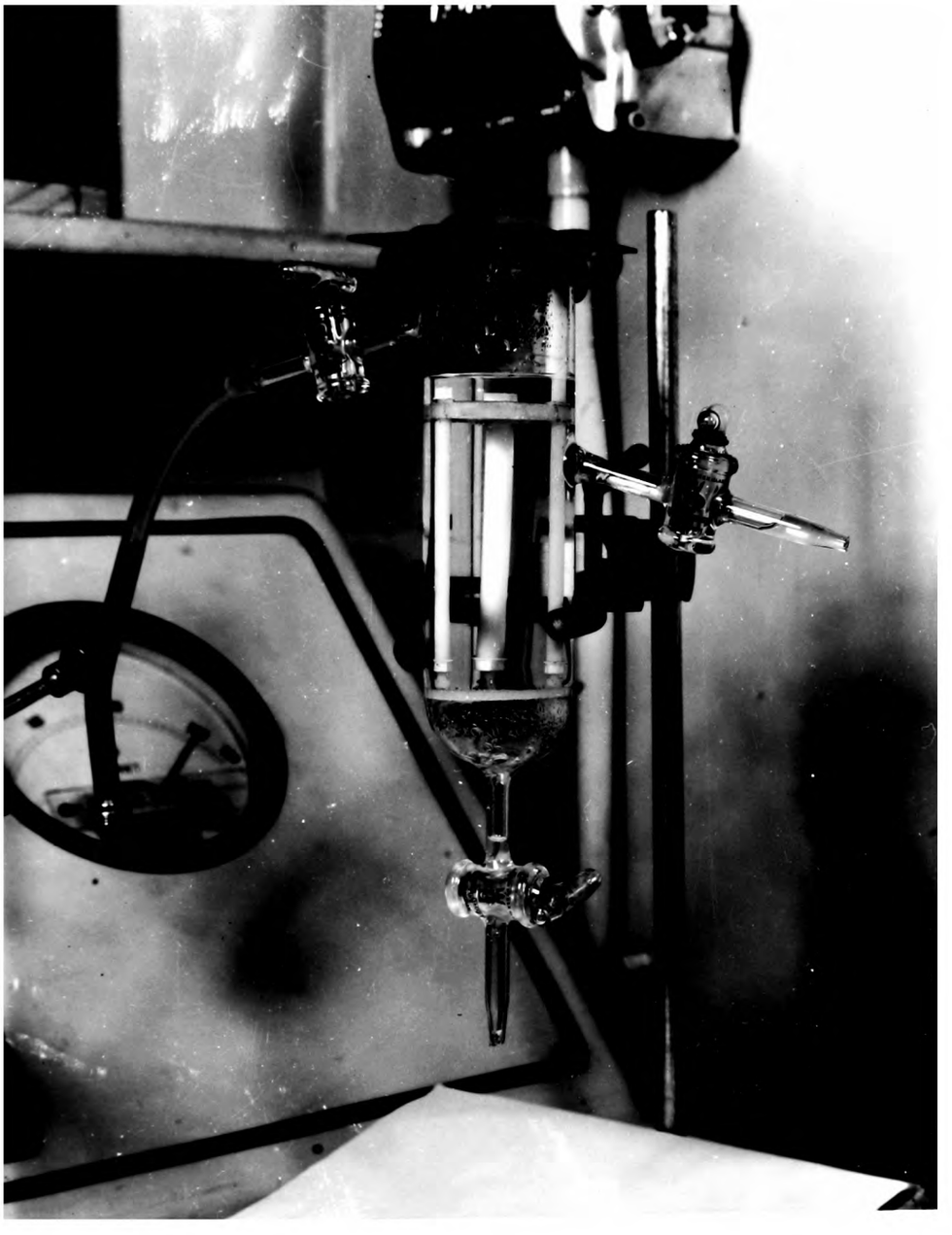

Figure AI-2

The nylon jig in the irradiation vessel

bleeder valve, through a flow meter (q.v. figure AI-3), and through a curved steel tube which could be inserted into a Hersch cell (Hersch, 1957a, b) (q.v. figure AI-4).

Gassing with white spot nitrogen at a rate of 40 ml/min reduced the oxygen concentration of the outflow gas[1] to nearly that of the flushing gas after 20 minutes. The background oxygen concentration in the Hersch cell was usually $\sim 1 \text{ ppl0}^6$. A graph of oxygen clearance rate with gassing time is presented in figure AI-5. In the experiments reported in this addendum, gas flow rates in excess of 40 ml/min were used and gassing was continued, in all cases, until an oxygen concentration of $\sim 10 \text{ ppl0}^6$ or less was obtained.

When gassing was completed, the cylinder valve was shut, the water level adjusted (lowered) to a level of 9.0 mm above the upper layer of bolting cloth, and all three valves were shut. The inflow and outflow connections were removed and the sealed vessel was placed and centred underneath the X-ray tube head (q.v. figure AI-6). The apparatus was irradiated from above with a Resomax machine (300 kVp, 2 mm Cu H.V.L., 19.5 mA, 170 rad/min) adjusted so that the distance from the target to the miniscus of the water was 360 $\pm$ 1 mm. The X-ray beam was columnated through an 11 cm diameter apperture diaphragm of 6 mm Pb + 2 mm brass.

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1. For experiment 4014 this was 6.5 $\text{ppl0}^6 O_2$; for 5614, $\sim 2 \text{ ppl0}^6 O_2$; and for 7914, $\sim 10 \text{ ppl0}^6 O_2$.

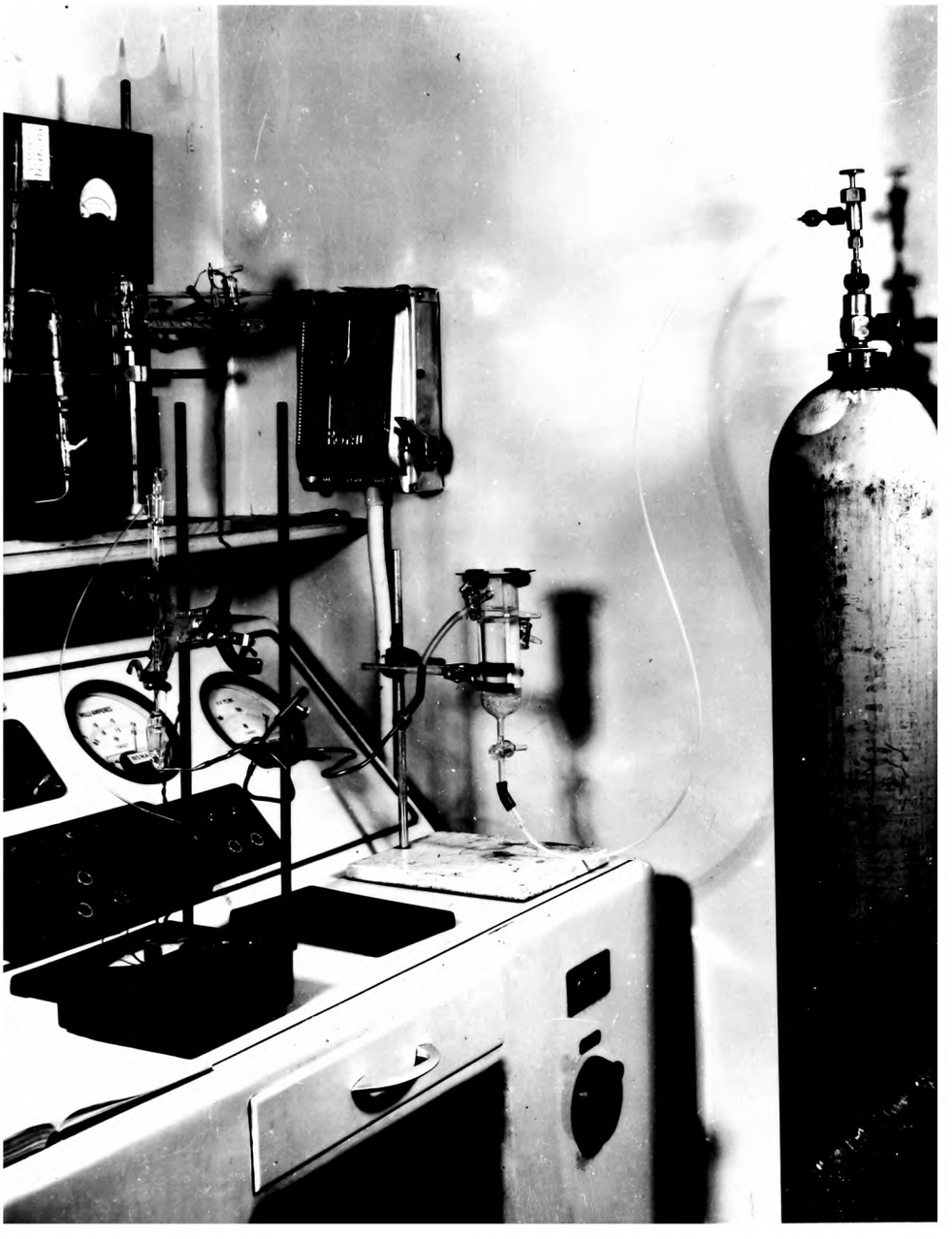

Figure AI-3

The gas flow setup

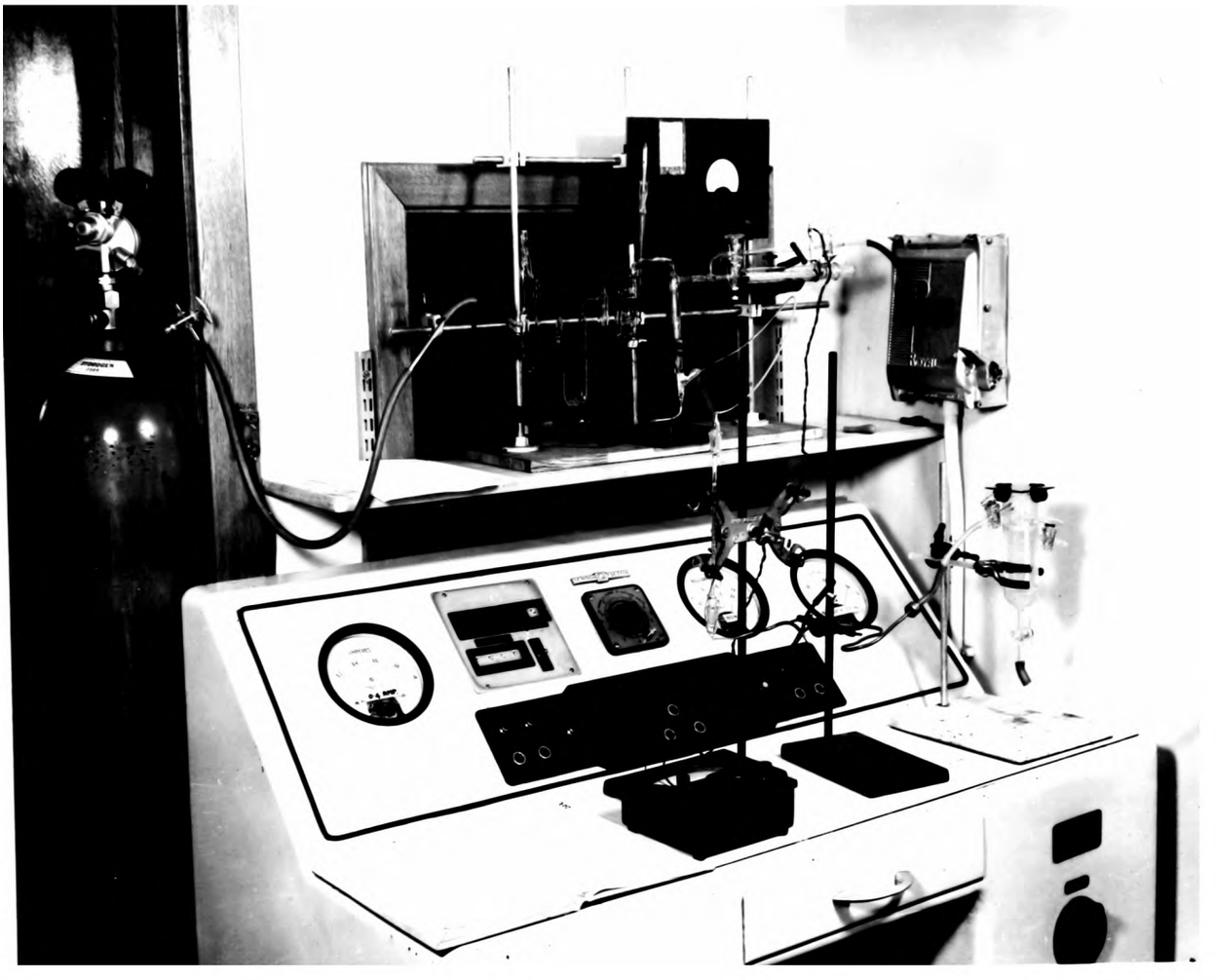

Figure AI-4

The gas flow setup and the Hersch cell

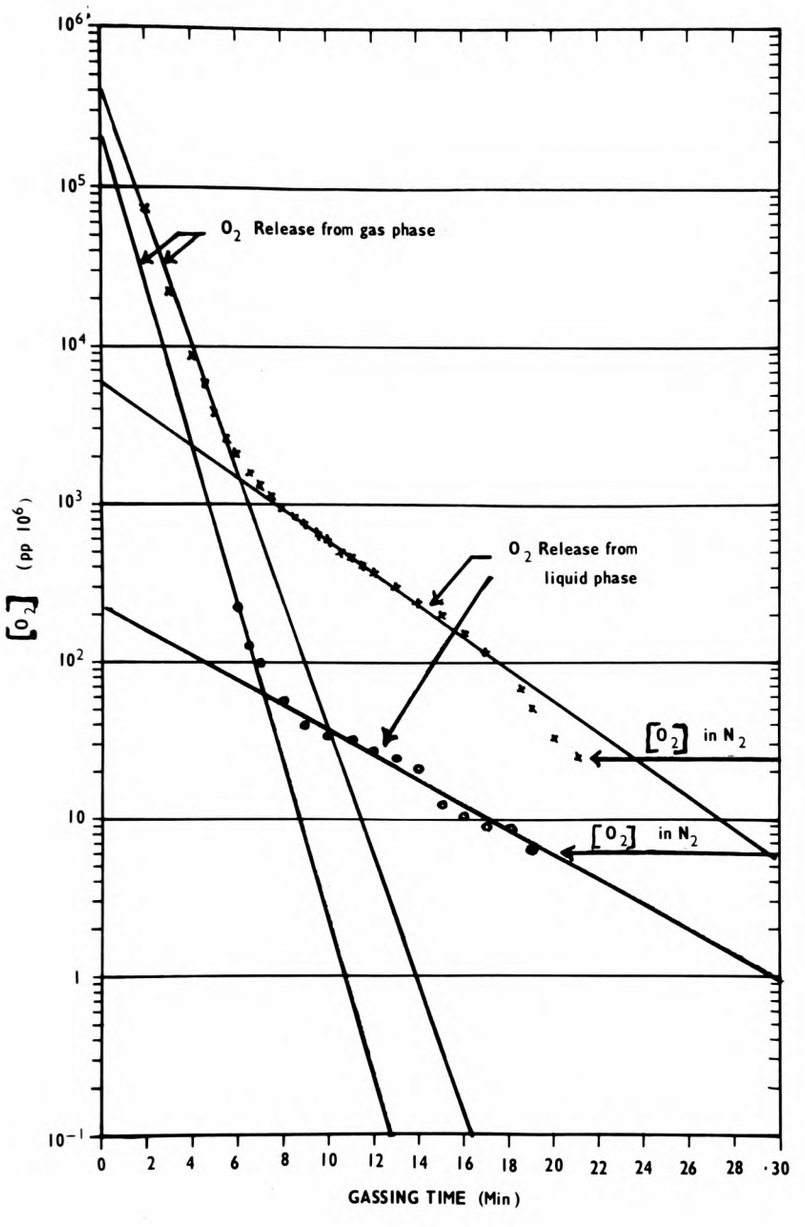

Figure AI-5

Rates of O_2 clearance

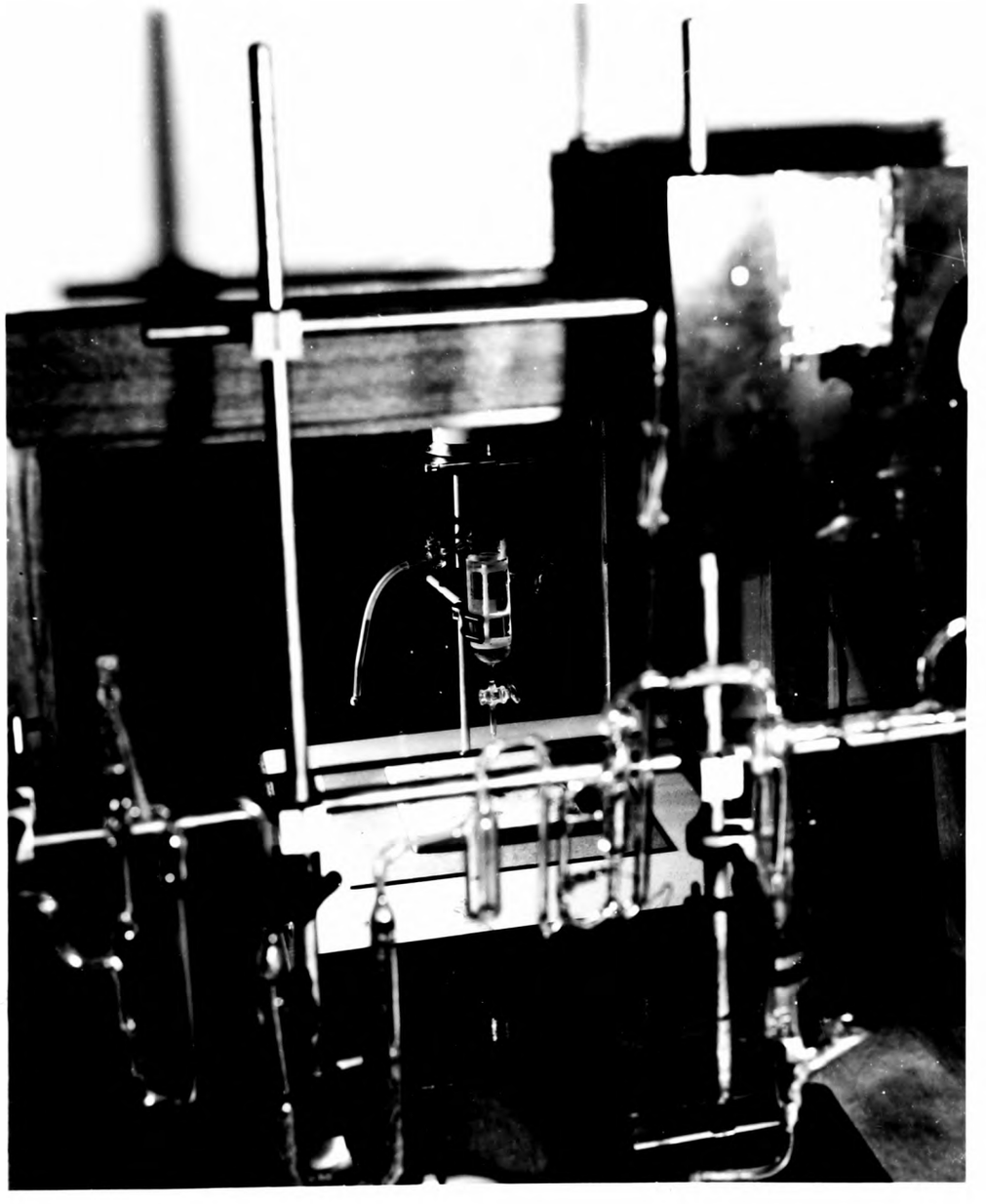

Figure AI-6

The irradiation vessel in position under the X-ray tube head

Results

The data were treated as described in chapter V A and B, and the resultant animal survival curve parameters with their standard errors of estimation are presented in table AI-1. In no case were the inverse slopes of the hypoxic survival curves significantly different from those of the oxic survival curves[1], but the LD_{50}'s for conditions of hypoxic irradiation were in every case, highly significantly greater than those for oxic conditions ($P < 0.01$). Thus planarian radiosensitivity is subject to an "oxygen effect", with oxygen enhancement ratios (O.E.R.)[2] of $2.90^{3} \pm 0.44$ (P. felina) and 2.54 ± 0.25 (D. lugubris B). Furthermore, the animal survival curve parameters under oxic irradiation conditions were not significantly different from those of the "population standards" of chapter IV D. Thus we conclude that the standard conditions of irradiation for the experiments reported in this thesis were not conditions of hypoxia.

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1. The discussion of the limitations of the applicability of inverse slopes to D_o measurements presented in Appendix II holds for these data as well. However, the value N of E may not be independent of the degree of anoxia.

2. $\text{O.E.R.} = \dfrac{LD_{50}(N_2)}{LD_{50}(\text{Air})}$

3. For the combined data of experiments 56 and 79. The LD_{50}'s for both oxic and hypoxic groups in experiment 56 are significantly different from those obtained in experiment 79; however, the O.E.R.'s calculated for each experiment are not significantly different from each other or from that derived from the combined data of both experiments.

Table AI-1

Animal Survival Curve Parameters for Irradiations in Nitrogen and in Air

Species	Gas	LD_{50} ± S.E.	K ± S.E.	C ± S.E.	J	Experiment
Polycelis felina	N_2	5104 ± 167	728 ± 189	3.1 ± 2.6%	5	5614
	N_2	3455 ± 141	1380 ± 1130	45 ± 32%	4	7914
	N_2	4447 ± 518	1128 ± 511	12.6 ± 14.5%	12	5614 + 7914
	Air	1954 ± 99	535 ± 118	0 ± 2.7%	6	5601
	Air	1131 ± 119	349 ± 102	0 ± 3.2%	4	7901
	Air	1535 ± 148	583 ± 176	0 ± 4.2%	13	5601 + 7901
	Air*	1650 ± 126	508 ± 137	8.4 ± 7.4%	36	Pop.Std.**
D.lugubris B	N_2	4261 ± 193	696 ± 267	0 ± 2.6%	4	4014
	Air	1677 ± 143	936 ± 206	12.0 ± 5.5%	49	Pop.Std.**

* Ungassed tap water (set up No.29)

** q.v. chapter V D. Population Standard.

Appendix II

R.B.E. of $^{90}Sr/^{90}Y$ β^- rays with Respect to 300 kVp X-rays in D. lugubris A and B

Appendix II

R.B.E. of $^{90}Sr / ^{90}Y$ β^- rays with respect to 300 kVp X-rays in D. lugubris A and B

Inspection of the inverse slopes (K's) of the computer fitted planarian survival probit regression lines(with their standard errors for reliability of estimation), allows one to conclude that there is no significant difference between the inverse slopes of diploid (A) and triploid (B) or of X- and β^- ray probit whole animal survival curves (q.v. table AII-1). Thus if these four groups of animals do derive from different populations, the accuracy of the inverse slope determinations does not allow this to be apparent. As K is a measure of the variance of the animals at risk, with respect to survival after irradiation, and as there is no evidence that the value of K differs for each of the four groups; then, if the mean (LD_{50}) of each of these four groups does not differ, we can say that we are dealing with samples of a homogeneous population (with respect to survival).

We remember that according to our simple model:

$$D_o = K / 1.2 = 0.833K$$

and

$$LD_{50} = D_o \left[\ln(N_{\alpha E}) + 0.65\right],$$

so that a ΔD_o of, say, +12% would increase K by less than 14%. A change of such magnitude is much smaller than the accuracy

to which K can be experimentally determined under our laboratory conditions, whereas the LD_{50} would (for NαE = constant) show a magnification of the order of a factor of two and four, i.e. a change of 24% and 48% (for triploids and diploids respectively). LD_{50} changes of this order are detectable. Thus, since the LD_{50} is a statistic of much greater accuracy of determination than K, and as we do not expect the values of N, α, or E to change radically with irradiation, if the LD_{50}'s of the four groups differ, then it is most likely that these differences are due to changes in D_o (for the neoblasts). Estimates of the relative biological efficiency (R.B.E.) of $^{90}Sr/^{90}Y$ β^-rays to 300 kVp X-rays for diploid and triploid D. lugubris, based on the LD_{50} data given in table AII-2, are presented (with their standard errors) in table AII-3.

The β^-ray/X-ray R.B.E.'s are significantly different from 1.0 at the 95% and 99% fiducial levels for diploids and triploids respectively, but the R.B.E. values found for diploids and triploids are not significantly different from each other (by the "t-test" after an "F" ratio for equality of variances test to confirm the applicability of the "t-test").

Table AII-1

Inverse Slope of Survival Curves for Diploid and Triploid D. lugubris after X-rays and β^- rays

Ploidy \ Radiation Quality	X-Ray				β^- Ray			
	K	S.E.	Var	J	K	S.E.	Var	J
2n	873	191	3.6481×10^4	18	595	244	5.9312×10^4	27
3n	936	206	4.2436×10^4	49	967	270	7.2900×10^4	27

Table AII-2

LD_{50} of Diploid and Triploid *D. lugubris* after X-rays and β^- rays

Ploidy \ Radiation Quality	X-Ray				β^- Ray			
	LD_{50}	S.E.*	Var	J	LD_{50}	S.E.*	Var	J
2n	2674	177	3.1329×10^4	18	3320	176	3.0814×10^4	27
3n	1677	143	2.0449×10^4	49	2472	200	4.0000×10^4	27

* S.E. of LD_{50} estimation; S.E. for all rad doses quoted = ± 3% (= R.M.S. of 2% variation + 2% deviation from N.I.L. standard).

Table AII-3

R.B.E. of $^{90}Sr/^{90}Y\beta^-$ rays to 300 kVp X-rays in Diploid and Triploid D. lugubris

Ploidy	R.B.E.*	S.E.	Var	ν
2n	0.805	0.068	4.65_{-3} x 10^{-3}	45
3n	0.678	0.080	6.358_{-3} x 10^{-3}	76

$$* \text{ R.B.E.} = \frac{LD_{50}(\text{X-ray})}{LD_{50}(\beta^- \text{ray})} = \text{R.B.E.}\left(\frac{\beta^- \text{ray}}{\text{X-ray}}\right)$$

Appendix III

A Correction to Abercrombie's Formula

Corrections for Multiple Counting

Appendix III

Corrections for Multiple Counting

The pitfalls of extrapolating from nuclear or cellular densities in one microtome section to the mean number within one mm^3 or any given mass of tissue, were discussed by Abercrombie (1946). In short, he showed that in any section some of the cells - nuclei etc. - are cut and appear in more than one of two or more adjacent serial sections, but are not recognised as being cut. Thus the number of cells, or objects, counted in a section represents more than just those cells, the centres of which lie in that section. As the cell diameter approaches the section thickness, this overestimate increases. He derives a simple correction formula which is valid for any shape of object randomly distributed relative to the section position and thickness.

This formula is:

$$P = A \frac{M}{(L+M)}$$

where P is the average number of objects (cells) with centres in the section, A is the crude count of the number of objects seen in the section, M is the thickness (inμ) of the section, and L is the average length (inμ) of the objects. Thus for 10μ objects in 10μ sections, the overestimate is 100%. When comparing relative densities of different objects, the results

will depend on the section thickness unless this correction formula is applied to <u>both</u> sets of crude counts.

The L in Abercrombie's formula is the mean true length (or diameter for spherical objects) which is different from the mean measured length when length is measured in sections. He implicitly assumes that all objects are cut and therefore takes the mean measured diameter as the mean chord of a circle or $\frac{\pi}{4}$ times the true diameter. Although he argues that the error of density overestimation due to use of the mean measured diameter, in practice, seldom exceeds 10%, the author thinks that it may be useful to consider a more rigorously derived formula for obtaining the mean true diameter from the mean measured diameter, valid for any object length and section thickness. This would serve the twofold purpose of enabling one to obtain mean true diameters (or lengths) from measurements based on sectioned material (if the objects are randomly distributed relative to the section thickness), and would allow one to use Abercrombie's formula without introducing errors, albeit small ones, due to poor estimation of object length.

Consider a section of thickness "T" taken through a block of substance containing objects of diameter D_t (see figure AIII-1). All objects with centres lying between points A and D will be counted as lying in section T and will thus contribute to the mean measured diameter. Those objects with

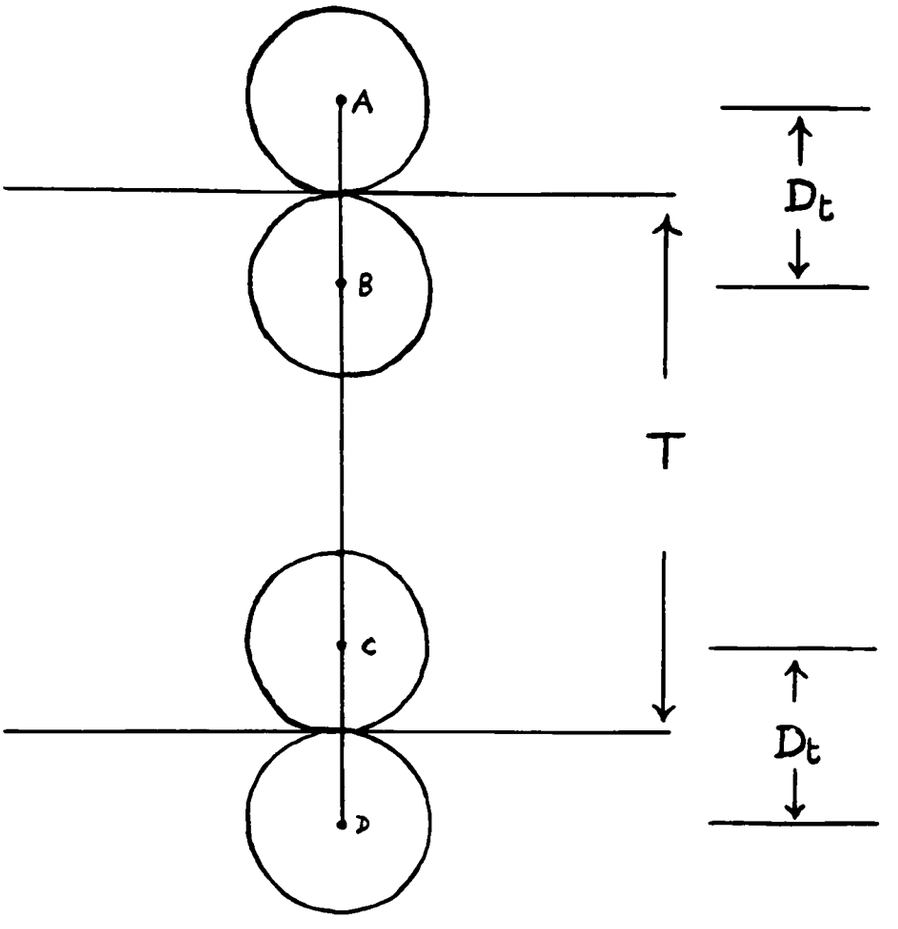

Figure AIII-1

Diagram to illustrate derivation of equation relating mean measured diameter of objects seen in serial sections to their mean true diameter

A, B, C and D = centres of spheres of radius $D_t/2$. T = thickness of section.

centres between B and C will be uncut and each will contribute a length D_t (the true diameter) to the mean measured diameter (D_m). The proportion of objects counted in the section T, having centres between B and C, is $\frac{BC}{AD}$ or $\frac{T-D_t}{T+D_t}$. All objects appearing in section T with centres not between B and C will be cut and each will contribute, on average, $\frac{\pi}{4} D_t$ to the mean measured diameter (mean chord of a circle = $\frac{\pi}{4}$ x diameter). The proportion of cut objects is 1- /the proportion of uncut objects/ so we can write:

$$D_m = \left(\frac{T-D_t}{T+D_t}\right) D_t + \frac{\pi}{4} D_t \left(1 - \frac{T-D_t}{T+D_t}\right)$$

or

$$D_m = \left(\frac{T-D_t}{T+D_t}\right) D_t + \frac{\pi}{4} D_t \left(\frac{2D_t}{T+D_t}\right)$$

which can be expressed as a quadratic equation in D_t:

$$D_t^2 \left(\frac{\pi}{2} - 1\right) + D_t \left(T-D_m\right) - TD_m = 0$$

which has as its solution:

$$D_t = \frac{-(T-D_m) \pm \sqrt{(T-D_m)^2 + (2\pi-4)\, TD_m}}{(\pi-2)} .$$

Thus the mean true length of any normally distributed object can be determined from the mean length as measured in sectioned material, and the section thickness.

Measurements of nuclear diameters (taken as the average

of two measurements at right-angles to each other, for each cell) were made for both diploid and triploid neoblasts. A sample of twenty-five diploid, and thirty-eight triploid, neoblasts produced mean measured diameters of 5.6± 0.8 and 5.8 ± 1.1, corresponding to mean true diameters of 6.8 (6.3-7.2, 95% fiducial limits) and 7.0 (6.5-7.6, 95% fiducial limits)μ respectively, for fixed cells, clearly not a significant difference.

However, for the conditions and data presented here, the difference in the Abercrombie correction factor for taking $\frac{4}{\pi} D_m$ instead of D_t, amounts to only 1%. Thus:

$$\frac{M}{L+M} = \frac{10}{16.8} = 59.5\% \text{ for diploids}$$

$$= \frac{10}{17.0} = 59.2\% \text{ for triploids.}$$

Therefore the comparisons between diploids and triploids in chapter VII part C are not affected by this correction as both correction factors are in agreement within the experimental error. The comparison in chapter VII part B is also valid but the "number of neoblasts" scale should be shifted to the right, as shown in the lower scale, so as to give the true number instead of the absolute count. The importance of this for the comparison of work performed in different laboratories can be seen in chapter VII.

Appendix IV

Spontaneous Tumours in *D. etrusca* and *D. ilvana*

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Observations on some tumours found in two species of planaria—*Dugesia etrusca* and *D. ilvana*

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Since the description by Pallas in 1774 of their remarkable capacity for regeneration the planarians have been of interest to the biologist (cf. Brøndsted's excellent review—Brøndsted, 1955). Dubois (1949) has shown that regeneration in planaria is due to undifferentiated totipotential cells referred to as neoblasts, which are the only cells known to divide mitotically. When the animal is cut or similarly injured, some neoblasts in the region of the wound rapidly differentiate to form a new epidermis, while other neoblasts divide and migrate towards the wound surface where they continue to divide and/or differentiate into various cell types. The finding of spontaneous growths or tumours in this simple material (Goldsmith, 1939) suggests that it may be of interest to study the nature of such tumours.

MATERIALS AND METHODS

Material for the present studies includes the species *Dugesia etrusca* and *D. ilvana* obtained from the laboratory of Professor Mario Benazzi of the University of Pisa, Italy. *D. etrusca* was collected in Toscana and *D. ilvana* from Isola d'Elba. Both species are diploid. The animals, ten of each species, were kept individually or in pairs in 13 ml. polystyrene tubes (Camlab) filled with tap water and maintained at 20 ± 1 °C. They were fed on washed slices of liver (beef, sometimes lamb) twice weekly. The water was changed, and waste matter removed, 24 h after feeding. Under these conditions both species multiply rapidly by transverse fission. Progeny were placed in separate tubes as often as was necessary to prevent overcrowding. After 17 weeks the ten animals of *D. etrusca* and *D. ilvana* had multiplied to ninety-four and eighty-eight respectively, and at that time eighteen and twelve animals, respectively, were found to have tumorous growths (Text-fig. 1). The animals were therefore isolated and the progress of the tumours was followed. Weekly checking of the normal population showed further examples of such tumours. A total of fifty-four tumour-bearing animals was studied. No difference was observed between the tumours of

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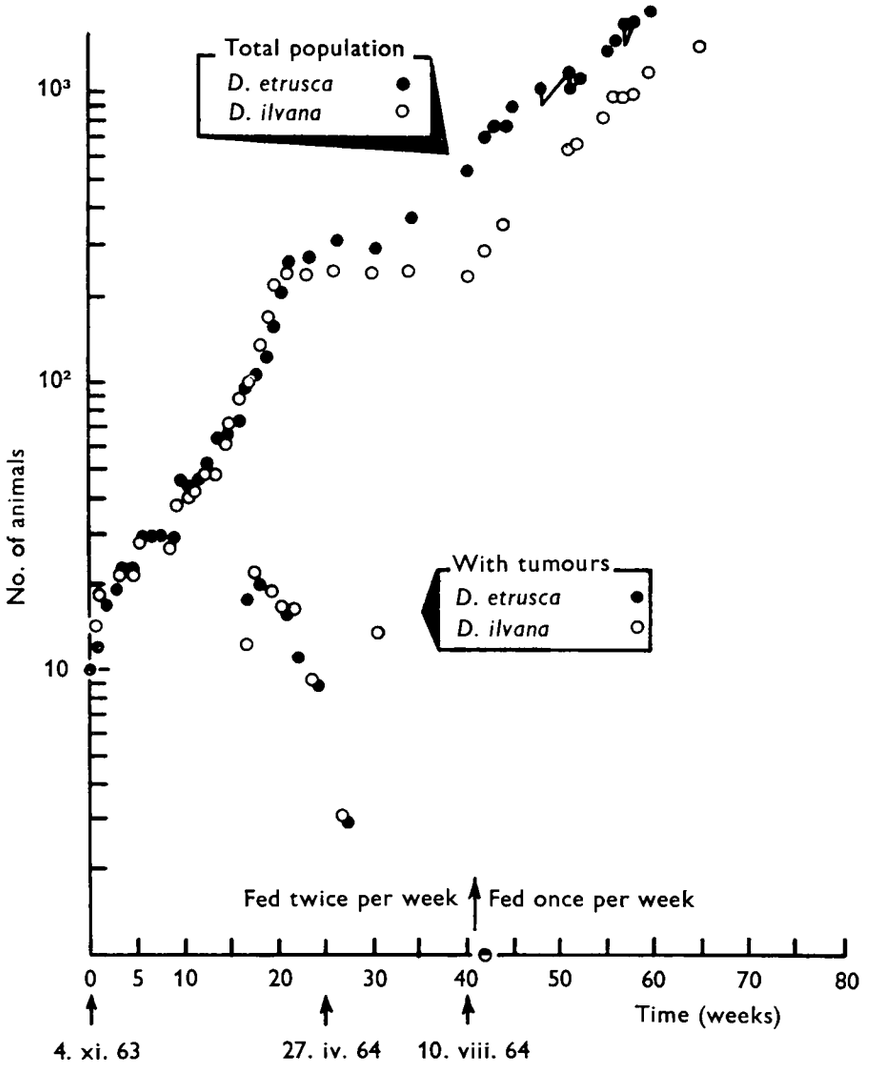


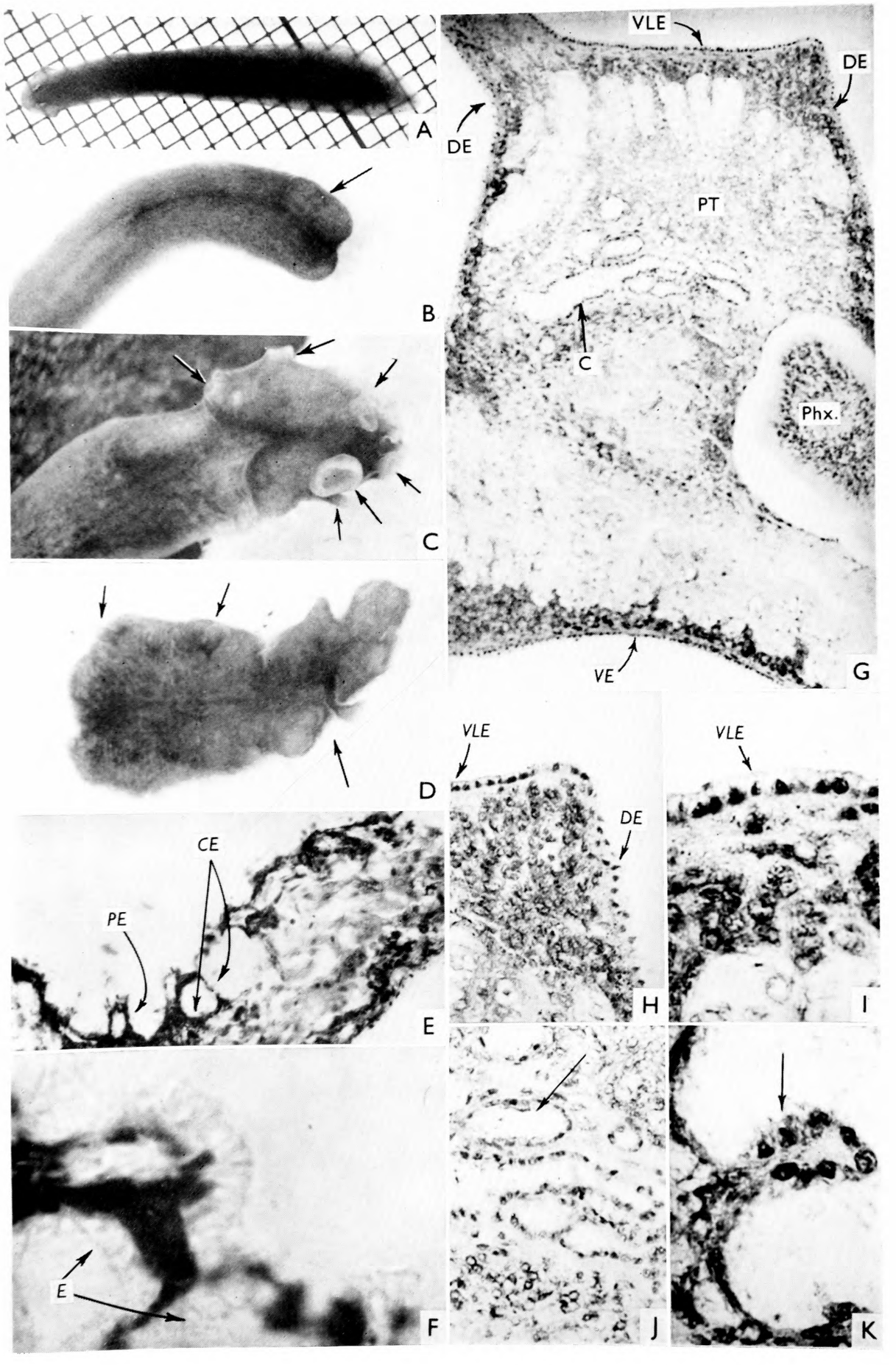
Text-fig. 1. Rates of population increase and appearance of growths.

D. etrusca and *D. ilvana*, and they can be described together. *D. ilvana* is indistinguishable from *D. etrusca* on the basis of body shape (Plate 1, fig. A).

OBSERVATIONS

Most of the tumours began in the posterior tip, appearing as a reddened (normal pigmentation is a tan or slightly red-tan) swelling or lump or group of lumps which stretched the skin and distorted the shape of the tail (Plate 1, fig. B). The tail became very much thicker and impeded the planarian's movement. The lumps grew slowly, became darkly pigmented, and eventually developed clear, white, tubes from which mucus was at times expelled (Plate 1, fig. C).

The planarians, which were rapidly growing and increasing in number, continued to divide even after the tumour had become the largest part of the body. The anteriors gave rise to healthy animals in which recurrence of the tumours has not yet been observed (1 year); while the posterior portion retained the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tumour. The posterior portion regenerated a new head at the normal rate and divided again, leaving a smaller posterior piece containing the tumour (Plate 1, fig. D). Sometimes the second division took place before the 'posterior' head was completely regenerated; however, this occasionally happened in the normal animal as well. Finally, the posterior portion as well as the tumour lysed.

In one case (*D. etrusca*) a planarian was found with growths on both the anterior and posterior ends. No fission occurred and the mid-region shrank rapidly until final lysis of both this region and the tumours occurred.

After cutting, normal tissue is regenerated whereas the tumour tissue is not. After a cut through normal tissue, the planarian's muscles contract, pinching off the wounded area and thus preventing cells from pouring out into the surrounding environment. Local neoblasts differentiate to form a new epithelium over the wound surface. Finally, a stimulation occurs in other nests of neoblasts as more cells are needed, and the normal regenerative phenomena follow. However, after a cut through the tumour, the pinching off of the wounded area did not occur. Cells streamed out until all the tumour tissue, as well as a large quantity of normal tissue, had leaked out. Then the pinching off occurred, well beyond the original boundaries of the tumour, and normal regeneration followed.

When the animals were reduced to one feeding per week no new occurrences of tumours were found in the population as a whole in my material (apart from two examples which are described below and which were first observed one week after feeding had been reduced to once a week).

In two cases, the tumour started in the mid-region of the dorsum just posterior to the mouth and assumed a wide crater-like shape with a clear ring of tissue forming a rim around the apex of the mound. In both cases the planarians did not divide and more tumours appeared on the posteriors, which soon afterwards began to lyse. At this point both animals were fixed for histological study. Morphologically, the crater-like tumours appeared very much like the advanced stages of the other tumours described, only being somewhat larger and perhaps more advanced. Their position immediately posterior to the pharynx may be the reason for the anteriors not dividing away from the tumours (fission normally takes place through this region).

Histological preparations of tumour-bearing animals show an enlarged tail thickness, consisting mainly of papillary and crypt-like structures covered and lined, respectively, by mucus-producing epithelium (Plate 1, figs. E, F); and a broken or perforated basement membrane. The epithelial lining of the crypt-like structures is typical of most of the tumours described at the beginning of this paper.

The crater-like tumour is notable for its large mass, the high degree of differentiation of some of its tissues (e.g. nervous, parenchymal and epithelial tissues), and its epithelium-lined crypts (Plate 1, figs. J, K). Plate 1, fig. G, shows such a tumour which occurred in the mid-region of the animal just posterior to the mouth; the posterior portion had already lysed (the tumour there was more

like the first-described tumours). The top of the tumour is lined with a ventral-like epithelium (Plate 1, figs. H, I).

In methyl-green pyronin-stained sections, large pockets of neoblasts sprinkled about the tumour are visible. At higher magnifications of an epithelium-lined crypt, the epithelial lining is not unlike the ventral epithelium (Plate 1, figs. H, I, J, K), and the ventral-like epithelium-lined tumour surface may have been derived from the opening-up (bursting?) of such a crypt. This might be a similar phenomenon to that observed in embryos of *Polycelis tenuis* (Skaer, 1965), where vesicles of ciliated cells migrate to the ventrum and open to form the ventral epithelium.

The cells and tissues found in these tumours do not appear to be abnormal in themselves, but rather in their position with respect to their surrounding tissues and the rest of the animal.

CONCLUSIONS AND DISCUSSION

Other workers have reported abnormal growths in planarians. Melander's description (Melander, 1950) of growths associated with the occurrence of accessory chromosomes in *Polycelis tenuis* is totally unlike the tumours which

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PLATE 1

Fig. A. Normal planarian (*Dugesia ilvana*). Each square is 0·9 mm a side. Posterior on right, anterior on left. (× 2·7.)

Fig. B. Posterior portion of a tumour-bearing planarian. This is the first visible stage in the development of the growth. The arrow shows the dorsal epithelium stretched over the lump. (× 3·3.)

Fig. C. Differentiated stage of the growth. The clear white tubes are indicated by the arrows. (× 4·8.)

Fig. D. Final stage of the growth. Rapid, complete lysis occurs shortly after this stage. The arrows show that the growth (tubes) has reached the anterior edge (left) of this small posterior piece. (× 5.)

Fig. E. Posterior portion of a sagittal section of a tumour-bearing planarian. Methyl-green pyronin, × 204. *CE*, Epithelium-lined crypt; *PE*, epithelium-lined papillary structure. Dorsum on upper left, ventrum on lower right corner.

Fig. F. Enlargement of part of fig. E (× 892). *E*, Epithelial lining of crypt.

Fig. G. Sagittal section of the crater-like growth, showing the differentiation of the tissues, i.e. the epithelium-lined crypts (*C*), parenchymal tissue (*PT*), the dorsal (*DE*) and ventral-like (*VLE*) epithelia covering, respectively, the sides and top of the tumour. Haematoxylin, × 112. *Phx.*, Pharynx; *VE*, ventral epithelium.

Fig. H. Enlargement of part of fig. G, showing the dorsal epithelium (*DE*) on the anterior facing rise of the growth and the ventral-like epithelium (*VLE*) covering the top (dorsal surface) of the growth. Haematoxylin, × 254.

Fig. I. A further enlargement, showing the ventral-like epithelium (*VLE*) covering the dorsal-facing top of the tumour. Compare with fig. K. Haematoxylin, × 605.

Fig. J. The epithelium-lined crypts. Haematoxylin, × 245. Compare with fig. H.

Fig. K. Higher magnification, showing part of an epithelium-lined crypt. Compare with Fig. I. Haematoxylin, × 617.

I have observed. Stéphan (1960) gives a detailed description of tumours in *D. tigrina*, first described by Goldsmith (1939, 1941), which although morphologically similar in many ways to the tumours found in *D. etrusca* and *D. ilvana*, differ strikingly from them. In *D. tigrina*, tumours recurred with a high frequency. Furthermore, in no case of mine did the tumours differentiate into heads or tails, as reported by Stéphan (1960) for *D. tigrina*.

D. etrusca and *ilvana* are localized species of the '*gonocephala*' group and are classified as *D. etrusca* Benazzi and *D. ilvana* Benazzi.

The literature also contains several reports of induction of planarian tumours, but Foster (1963) is the only one to suggest that these growths are neoplastic. It is also possible that these tumours are the result of faulty differentiation control—a not unlikely possibility, as differentiation of the totipotential neoblast is determined by the surrounding non-mitotic somatic tissue (Dubois, 1949; Lender, 1960: Kolmayer & Stéphan-Dubois, 1960; Stéphan-Dubois, 1961; Stéphan-Dubois & Gilgenkrantz, 1961).

A further study of growths of this kind may be particularly instructive on aspects of oncogenesis not seen in animals with a low capacity of regeneration.

SUMMARY

Spontaneously differentiated tumours in planaria which had been multiplying rapidly by transverse fission are described. Development was progressive, leading to lysis of both tumour and host. No recurrence was observed in anteriors of animals which had divided away from tumour-bearing posteriors, nor were new occurrences observed in slower-growing animals fed once, instead of twice, per week. It is suggested that the tumours might be the result of differentiation error rather than neoplasia.

RÉSUMÉ

Observations sur quelques tumeurs découvertes chez deux espèces de Dugesia

On décrit des tumeurs d'origine spontanée chez des planaires qui s'étaient multipliées rapidement par scissiparité. Le développement en était progressif, aboutissant à une lyse conjointe de l'hôte et de la tumeur. On n'a pas observé de récidive dans des régions antérieures d'animaux qui s'étaient séparées de régions postérieures porteuses de tumeurs, et il n'y a pas eu non plus de nouvelles apparitions chez des animaux à croissance lente, nourris une seule fois par semaine au lieu de deux fois. On suggère que les tumeurs pourraient être le résultat d'une 'erreur' de la différenciation plutôt que d'une néoplasie.

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REFERENCES

BRØNDSTED, H. V. (1955). Planarian Regeneration. *Biol. Rev.* **30**, 65–126.

DUBOIS, F. (1949). Contribution à l'étude de la migration des cellules de régénération chez les Planaires dulcicoles. *Bull. biol. Fr. Belg.* **83**, 215–83.

FOSTER, J. A. (1963). Induction of neoplasms in planarians with carcinogens. *Cancer Res.* **23**, 300–3.

GOLDSMITH, E. D. (1939). Spontaneous outgrowths in *Dugesia tigrina* (Syn. *Planaria maculata*). *Anat. Rec.* **75** (suppl.), 158–9.

GOLDSMITH, E. D. (1941). Further observations of supernumerary structures in individuals of an artificially produced clone of *Dugesia tigrina*. *Anat. Rec.* **81** (suppl.), 108–9.

KOLMAYER, S. & STÉPHAN-DUBOIS, F. (1960). Néoblasts et limitation du pouvoir de régénération céphalique chez la Planaire *Dendrocoelum lacteum*. *J. Embryol. exp. Morph.* **8**, 376–86.

LENDER, TH. (1960). L'Inhibition spécifique de la différenciation du cerveau des Planaires d'eau douce en régénération. *J. Embryol. exp. Morph.* **8**, 291–301.

MELANDER, Y. (1950). Accessory chromosomes in animals, especially in *Polycelis tenuis*. *Hereditas*, **36**, 19–38.

SKAER, R. J. (1965). The origin and continuous replacement of epidermal cells in the planarian *Polycelis tenuis* (Ijima). *J. Embryol. exp. Morph.* **13**, 129–39.

STÉPHAN, F. (1960). Tumeurs spontanées chez la Planaire *Dugesia tigrina*. *C. r. Séanc. Soc. Biol.* **156**, 920–2.

STÉPHAN-DUBOIS, F. (1961). Les cellules de régénération chez la Planaire *Dendrocoelum lacteum*. *Bull. Soc. zool. Fr.* **86**, 172–85.

STÉPHAN-DUBOIS, F. & GILGENKRANTZ, F. (1961). Transplantation et régénération chez la Planaire *Dendrocoelum lacteum*. *J. Embryol. exp. Morph.* **9**, 642–9.

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Bibliography

LITERATURE CITED

ABELOOS, M., 1927. "La Vitesse de Régéneration de la Tête chez Planaria gonocephala Dugès. Influence de la Taille des Individus Opérés." C.r. Séanc. Soc. Biol., Paris, 96, 1923-4.

ABELOOS, M., 1930. "Recherches Expérimentales sur la Croissance et la Régénération chez les Planaires." Bull. biol. Fr. Belg., 64, 1-140.

ABELOOS, M., 1932. "La Régénération et les Problèmes de la Morphogenèse." Gauthier-Villars, Paris.

ABERCROMBIE, M., 1946. "Estimation of Nuclear Population from Microtome Sections." Anat. Rec., 94, 239-47.

ALEXANDER, P. and MIKULSKI, Z. B., 1961. "Differences in the Response of Leukaemia Cells in Tissue Culture to Nitrogen Mustard and to Dimethyl Myleran." Biochem. Pharmac., 5, 275-82.

ALLEN, G. D., 1919a. "Quantitative Studies on the Rate of Respiratory Metabolism in Planaria. I. The Effect of Potassium Cyanide on the Rate of Oxygen Consumption." Am. J. Physiol., 48, 93-120.

ALPER, T., 1961. "Effects on Subcellular Units and Free-Living Cells." In Mechanisms in Radiobiology, ed. Errera & Forssberg. Academic Press, N.Y. and London, 1961.

ALPER, T., GILLIES, N. E., and ELKIND, M. M., 1960. "The Sigmoid Survival Curve in Radiobiology." Nature, 186, 1062-3.

ANSEVIN, K. D., and BUCHSBAUM, R., 1961. "Observations on Planarian Cells Cultivated in Solid and Liquid Media." J. exp. Zool., 146, 153-7.

ATWOOD, K.C., VON BORSTEL, R. C., and WHITING, A. R., 1956. "An Influence of Ploidy on the Time of Expression of Dominant Lethal Mutations in Habrobracon." Genetics, Princeton, 41, 804-13.

BACQ, Z. M., and ALEXANDER, P., 1961. "Fundamentals of Radiobiology." 2nd Ed. Pergamon Press, Oxford, N.Y., London, Paris.

BARDEEN, C. R., and BAETJER, F. H., 1904. "The Inhibitive Action of the Roentgen Rays on Regeneration in Planarians." J. exp. Zool., 1, 191-5.

BEDFORD, J. S., and Hall, E. J., 1967. "Effect of Chromosome Complement on Radiation Dose Response and on Recovery from Radiation Damage." Br. J. Radiol., 40, 155.

BENAZZI, M., 1957. "Cariologia di Dugesia lugubris (O. Schmidt) (Tricladida Paludicola)." Caryologia, 10, 276-303.

BENAZZI, M., 1960a. "Ulteriori Ricerche sui Poliploidi Sperimentali della Planaria Dugesia lugubris (O. Schmidt)." Caryologia, 12, 414-38.

BENAZZI, M., 1960b. "Evoluzione Cromosomica e Differenziamento Razziale e Specifico nei Tricladi." Atti Accad. naz. Lincei Rc. Evoluzione e Genetica, 47, 273-97.

BENAZZI, M., 1961. "Il Comportamento dei Fattori di Pigmentazione in una Planaria Studiato Mediante le Variazioni di Ploidia (Nota Preliminare)." Atti Ass. genet. ital., 6, 291-2.

BENAZZI, M., 1962a. "L'Eredità della Pigmentazione nella Planaria Dugesia lugubris." Atti Ass. genet. ital., 7, 213-8.

BENAZZI, M., 1962b. "L'Azione delle Radiazioni Ionizzanti sullo Sviluppo delle Planarie." Atti Accad. naz. Lincei Rc., Ser. 8, 32, 26-9.

BENAZZI, M., 1963a. "Il Problema Sistematico delle Polycelis del Gruppo nigra-tenuis alla Luce di Ricerche Citologiche e Genetiche." Monitore zool. ital., 71, 288-300.

BENAZZI, M., 1963b. "L'Azione dello Spermio Irradiato nello Sviluppo Anfimittico e Pseudogamico delle Planarie." Atti Ass. genet. ital., 8, 255-6.

BENAZZI, M., 1965a. "Su. una Popolazione della Planaria Dugesia lugubris (Biotipo E)." Atti Accad. naz. Lincei Rc., Ser. 8, 38, 788-90.

BENAZZI, M., 1965b. "Personal communication."

BENAZZI, M., 1966. "Considerations on the Neoblasts of Planarians on the Basis of Certain Karyological Evidence." Chromosoma, 19, 14-27.

BENAZZI, M., and PUCCINELLI., I., 1961. "Analisi Comparativa del Cariogramma dei Biotipi di Dugesia lugubris." Atti Ass. genet. ital., 6, 419-26.

BENAZZI-LENTATI, G., 1943. "Sulla Reazione Metacromatica delle Cellule Indifferenziate del Parenchima di Alcune Turbellari." Monitore zool. ital., 54, 5-9.

BENDER, M. A., and GOOCH, P. C., 1962. "The Kinetics of X-ray Survival of Mammalian Cells in vitro." Int. J. Radiat. Biol., 5, 133-145 (1962).

BENNETT, C. A., and FRANKLIN, N. L., 1954. "Statistical Analysis in Chemistry and the Chemical Industry." John Wiley and Sons Inc.: New York and London.

BERGONIÉ, J., and TRIBONDEAU, L., 1906. "Interprétation de Quelques Résultats de la Radiotherapie et Essai de Fixation d'une Technique Rationnelle." C. r. hebd. Séanc. Acad. Sci., Paris, 143, 983-5.

BERKSON, J., 1944. "Application of the Logistic Function to Bio-Assay." J. Am. statist. Ass., 39, 357-65.

BERKSON, J., 1946. "Approximation of Chi-Square by 'Probits' and by 'Logits'." J. Am. statist. Ass., 41, 70-4.

BERKSON, J., 1949. "Minimum χ^2 and Maximum Likelihood Solution in Terms of a Linear Transform, with Particular Reference to Bio-Assay." J. Am. statist. Ass., 44, 273-8.

BEST, J. B., ROSENVOLD, R., SOUDERS, J., and WADE, C., 1965. "Studies on the Incorporation of Isotopically Labelled Nucleotides and Amino Acids in Planaria."

J. exp. Zool., 159, 397-403.

BLISS, C. I., 1934. "The Method of Probits - a Correction." Science, N.Y., 79, 409-10.

BOND, V. P., FLIEDNER, T. M., ARCHAMBEAU, J. O., 1965. "Mammalian Radiation Lethality - a Disturbance in Cellular Kinetics." Academic Press, N.Y. and London.

BRØNDSTED, H. V., 1939. "Regeneration in Planarians Investigated with a new Transplantation Technique." Biol. Meddr., 15, 1-39.

BRØNDSTED, H. V., 1942a. "Further Experiments on Regeneration-Problems in Planarians." Biol. Meddr., 14, 1-27.

BRØNDSTED, H. V., 1942b. "A Study in Quantitative Regeneration: the Regeneration of Eyes in the Planarian Polycelis nigra." Vidensk Meddr. dansk naturh. Foren., 106, 253-62.

BRØNDSTED, H. V., 1946. "The Existence of a Static, Potential and Graded Regeneration Field in Planarians." Biol. Meddr., 20, 1-31.

BRØNDSTED, H. V., 1947. "The Time-Graded Regeneration Field in Planarians." Proc. 6th Int. Congr. exp. Cytol., 585-7.

BRØNDSTED, H. V., 1953. "Rate of Regeneration in Planarians After Starvation." J. Embryol. exp. Morph., 1, 43-7.

BRØNDSTED, H. V., 1955. "Planarian Regeneration." Biol. Rev., 30, 65-126.

BRØNDSTED, A., and BRØNDSTED, H. V., 1952. "The Time-Graded Regeneration field in Planaria (Dugesia) lugubris."

Vidensk Meddr. dansk naturh. Foren., 114, 443-7.

BRØNDSTED, A., and BRØNDSTED, H. V., 1953. "The Acceleration of Regeneration in Starved Planarians by Ribo nucleic Acid." J. Embryol. exp. Morph., 1, 49-54.

BRØNDSTED, A., and BRØNDSTED, H. V., 1954. "Size of Fragments and Rate of Regeneration in Planarians." J. Embryol. exp. Morph., 2, 49-54.

BRØNDSTED, A., and BRØNDSTED, H. V., 1961. "Number of Neoblasts in the Intact Body of Euplanaria torva and Dendrocoelum lacteum." J. Embryol. exp. Morph., 9, 167-72.

BUCHANAN, J. W., 1933. "Regeneration in Phagocala gracilis (Leidy)." Physiol. Zoöl., 6, 185-204.

BYRON, J. W., 1967. "Enhancement of the Post-irradiation Protective Action of TAB by an α-Adrenergic Blocking Agent." Nature, Lond., 213, 635-6.

BYRON, J. W. and LAJTHA, L. G., 1966. "Radioprotection in Total-Body Irradiated Primates." Br. J. Radiol., 39, 382-5.

CHANDEBOIS, R., 1962. "Rôle des Éléments Fixes et Libres du Parenchyme dans la Régénération de Planaria subtentaculata (Drap.)." Bull. biol. Fr. Belg., 96, 203-27.

CHANDEBOIS, R., 1963a. "Cultures de Fragments de Planaires d'eau Douce." Annls. Épiphyt., 14, 141-52.

CHANDEBOIS, R., 1963b. "Action des Rayons X sur la Differentiation Cellulaire de Planaria subtentaculata." Bull. Soc. zool. Fr., 88, 632-44.

CHANDEBOIS, R., 1965a. "Cell Transformation Systems in Planarians", in Regeneration in Animals and Related Problems, ed. V. Kiortsis and H. A. L. Trampusch, North-Holland Publishing Co., Amsterdam.

CHANDEBOIS, R., 1965b. "Variations du Pouvoir Régénérateur de Planaires Irradiées en Fonction de la Dose de Rayons X." C. r. hebd. Séanc. Acad. Sci., Paris, 260, 4834-7.

CHEVTCHENKO, N. N., 1936. "Polaritätsumkehrung bei der Unter Drückung der Dominierenden Regionen bei Planaria." Zool. Anz., 115, 232-244.

CHILD, C. M., 1911a. "Experimental Control of Morphogenesis in the Regulation of Planaria." Biol. Bull. mar. biol. Lab., Woods Hole, 20, 309-31.

CHILD, C. M., 1911b. "Studies on Dynamics of Morphogenesis, etc. I. Physiological Dominance of Anterior over Posterior Regions in the Regulation of Planaria dorotocephala." J. exp. Zool., 11, 187-220, 221-80.

CHILD, C. M., 1915. "Individuality in Organisms." Chicago Univ. Press, Chicago.

CHILD, C. M., 1948. "Differential Oxidation and Reduction of Indicators in Reconstitution of Hydra and Planaria." Physiol. Zoöl., 21, 327-50.

CHILD, C. M., and WATANABE, Y., 1935. "The Head Frequency Gradient in Eupl. dorotocephala." Physiol. Zoöl., 8, 1-40.

COWARD, S. J., 1964. "Electrolyte Exchange in Regenerating Dugesia dorotocephala." Experientia, 20, 121-2.

COWARD, S. J., and FLICKINGER, R. A., 1965. "Axial Patterns of Protein and Nucleic Acid Syntheses in Intact and Regenerating Planaria." Growth, 29, 151-63.

COWARD, S. J., FLICKINGER, R. A., and GAREN, E., 1964. "The Effect of Nutrient Media upon Head Frequency in Regenerating Planaria." Biol. Bull. mar. biol. Lab., Woods Hole, 126, 345-53.

CURTIS, W. C., 1902. "The Life History, the Normal Fission and the Reproductive Organs of Planaria maculata." Proc. Boston Soc. nat. Hist., 30, 515-59.

CURTIS, W. C., 1928. "Old Problems and a New Technique." Science, N.Y., 67, 141-9.

CURTIS, W. C., 1936. "Effects of X-rays and Radium on Regeneration." Ch.12, pp.419-29 in Biological Effects of Radiation, vol.I, ed. B. M. Duggar. McGraw-Hill Book Co., Inc., New York and London.

CURTIS, W. C. and SCHULTZE, L. M., 1934. "Studies upon Regeneration. I. The Contrasting Powers of Regeneration in Planaria and Procotyla." J. Morph., 55, 477-512.

DEERING, R. A., and RICE, Jr., R., 1962. "Heavy Ion Irradiation of Hela Cells." Radiat. Res., 17, 774-86.

DONNELLY, G. M., and JAHN, A. H., 1964. "Radiosensitivity of the Salamander Desmognathus." Genetics, Princeton, 50, 244.

DUBOIS, F., 1948a. "Sur les Conditions de la Migration des Cellules de Régénération chez les Planaires d'eau Douce." C. r. Séanc. Soc. Biol., 142, 533-5.

DUBOIS, F., 1948b. "Démonstration de la Migration des Cellules de Régénération des Planaires par la Méthode des Greffes et des Irradiations Combinées." C. r. hebd. Séanc. Acad. Sci., Paris, 226, 1316-17.

DUBOIS, F., 1948c. "Sur une Nouvelle Méthode Permettant de Mettre en Evidence la Migration des Cellules de Régénération chez les Planaires." C. r. Séanc. Soc. Biol., 142, 699-700.

DUBOIS, F., 1949. "Contribution à l'Étude de la Migration des Cellules de Régénération chez les Planaires Dulcicoles." Bull. biol. Fr. Belg., 83, 213-83.

DUBOIS, F., 1950. "Continuité de la Migration des Néoblastes dans la Régénération de la Planaire Euplanaria lugubris." C. r. Seanc. Soc. Biol., 144, 1545-8.

ELKIND, M. M., 1960. "Cellular Aspects of Tumor Therapy." Radiology, 74, 529-41.

ELKIND, M. M., and SUTTON, H., 1959. "X-ray Damage and Recovery in Mammalian Cells in Culture." Nature, Lond., 184, 1293-5.

ELKIND, M. M., and SUTTON, H., 1960. "Radiation Response of Mammalian Cells Grown in Culture. I. Repair of X-ray Damage in Surviving Chinese Hamster Cells." Radiat. Res., 13, 556-93.

EMERY, E. W., 1967. "Personal Communication."

EVANS, H. J., and SPARROW, A. H., 1961. "Nuclear Factors Affecting Radiosensitivity. II. Dependence on Nuclear and Chromosome Structure and Organization." Brookhaven Symp. Biol., 14, 101-127.

FEDECKA-BRUNER, B., 1961. "La Régénération de l'Appareil Copulateur chez la Planaire Dugesia lugubris." Archs. Anat. microsk. Morph. exp., 50, 221-31.

FEDECKA-BRUNER, B., 1964. "Radiodestruction des Testicules Suivie de Regeneration chez la Planaire Dugesia lugubris." C. r. hebd. Séanc. Acad. Sci., Paris, 258, 3353-6.

FEINSTEIN, R. N., editor, 1963. Session 5 Discussion, "Implications of Organic Peroxides in Radiobiology." Radiat. Res., Suppl. 3 (1963), A.P., N.Y. and London.

FINNEY, D. J., 1947. "The Principles of Biological Assay." Jl. R. statist. Soc., Suppl., 9, 46-91.

FINNEY, D. J., 1949. "The Estimation of the Parameters of Tolerance Distributions." Biometrika, 36, 239-56.

FINNEY, D. J., 1962. "Probit Analysis." 2nd Ed. Cambridge Univ. Press, Cambridge.

FISHER, R. A., 1935. Appendix to Bliss, C. I.: "The Case of Zero Survivors." Ann. appl. Biol., 22, 164-5.

FLAX, M. H., and HIMES, M. H., 1952. "Microspectrophotometric Analysis of Metachromatic Staining of Nucleic Acids." Physiol. Zool., 25, 297-311.

FLETCHER, G. H., 1959. "Interpretation of Some Results of Radiotherapy and an Attempt at Determining a Logical Technique of Treatment." /A translation of the paper by Bergonié and Tribondeau (1906)/ Radiat. Res., 11, 587-8.

FLEXNER, S., 1898. "The Regeneration of the Nervous System of Planaria torva and the Anatomy of Double Headed Forms." J. Morph. Physiol., 14, 337-46.

FLICKINGER, R. A., 1963. "The Site of Incorporation of ^{14}C-Labelled CO_2 and Glycine in the Planarian Dugesia dorotocephala." Expl. Cell Res., 30, 605-7.

FLICKINGER, R. A., 1964. "Isotopic Evidence for a Local Origin of Blastema Cells in Regenerating Planaria." Expl. Cell Res., 34, 403-6.

FUNAIOLI, M. L., 1951. "Sul Problema Sistematico di Dugesia lugubris e Dugesia polychroa (O. Schmidt)." Memorie Soc. tosc. Sci. nat., 58, Ser. B, 77-84.

GADDUM, J. H., 1933. "Reports on Biological Standards. III. Methods of Biological Assay Depending on a Quantal Response." Spec. Rep. Ser. med. Res. Coun., Lond., No.183.

GILBERT, C. W., MULDAL, S., LAJTHA, L. G., and ROWLEY, J., 1962. "Time Sequence of Human Chromosome Duplication." Nature, Lond., 195, 869-73.

GINOZA, W., and NORMAN, A., 1957. "Radiosensitive Molecular Weight of Tobacco Mosaic Virus Nucleic Acid."

Nature, Lond., 179, 520-1.

GURNEY, C. W., LAJTHA, L. G., and OLIVER, R., 1962. "A Method for Investigation of Stem-Cell Kinetics." Br. J. Haemat., 8, 461-6.

GURNEY, C. W., and WACKMAN, N., 1961. "Impairment of Erythropoiesis by Irradiation." Nature, Lond., 190, 1017-8.

HALL, E. J., and CAVANAGH, J., 1967. "The Oxygen Effect for Acute and Protracted Radiation Exposures Measured with Seedlings of Vicia faba." Br. J. Radiol., 40, 128-33.

HALL, E. J., LAJTHA, L. G., and OLIVER, R., 1962. "X-ray Dose-Respose Relationship for Reproductive Integrity of Vicia faba." Br. J. Radiol., 35, 388-97.

HALL, E. J., OLIVER, R., SHEPSTONE, B. J., and BEDFORD, J., 1966. "On the Population Kinetics of the Root Meristem of Vicia faba Exposed to Continuous Irradiation." Radiat. Res., 27, 597-603.

HAMILTON, L., 1967. "A Comparison of the X-ray Sensitivity of Haploid and Diploid Zygotes of Xenopus laevis." Radiat. Res., 30, 248-60.

HAMMOND, C. W., 1954. "The Treatment of Postirradiation Infection." Radiat. Res., 1, 448-58.

HANSEN-MELANDER, E., MELANDER, Y., and REYNOLDSON, T. B., 1954. "A New Species of Fresh-Water Triclad Belonging to the Genus Polycelis." Nature, Lond., 173, 354.

HAYNES, R. H., 1964. "Molecular Localization of Radiation Damage Relevant to Bacterial Inactivation." Ch.5 in Physical Processes in Radiat. Biol. pp.51-69, L. Augenstein et al. (Eds.), Academic Press, N.Y.

HENDERSON, T. R., and EAKIN, R. E., 1959. "Irreversible Alteration of Differentiated Tissues in Planaria by Purine Analogues." J. exp. Zool., 146, 253-63.

HERSCH, P., 1957a. "Galvanic Oxygen Recorder. Part I." Instrum. Pract., 11, 817-23.

HERSCH, P., 1957b. "Galvanic Oxygen Recorder. Part II." Instrum. Pract., 11, 937-41.

HEWITT, H. B., and WILSON, C. W., 1959. "A Survival Curve for Mammalian Leukaemia Cells Irradiated in vivo (Implications for the Treatment of Mouse Leukaemia by Whole-Body Irradiation)." Br. J. Cancer, 13, 69-75.

HOFF-JØRGENSEN, E., LØVTRUP, E., and LØVTRUP, S., 1953. "Changes in Desoxyribonucleic Acid and Total Nitrogen in Planarian Worms During Starvation." J. Embryol. exp. Morph., 1, 161-6.

HORGAN, V. J., PHILPOT, J. St. L, PORTER, B. W., ROODYN, D. B., 1957. "Toxicity of Autoxidized Squalene and Linoleic Acid, and of Simpler Peroxides, in Relation to Toxicity of Radiation." Biochem. J., 67, 551-8.

HURSH, J. B., 1957. Univ. of Rochester, Report No.506, Oct. 1957. Cit. Bacq and Alexander, 1961.

HUTCHINSON, F., and NORCROSS, C., 1960. "Inactivation by Ionizing Radiation of Coenzyme A in Various Cells." Radiat. Res., 12, 13-19.

HUTCHINSON, F., and POLLARD, E., 1961. "Target Theory and Radiation Effects on Biological Molecules." In Mechanisms in Radiobiology, ed. Errera and Forssberg, Academic Press, New York and London, 1961.

HYMAN, L. H., 1919a. "On the Action of Certain Substances on Oxygen Consumption. II. Action of Potassium Cyanide on Planaria." Am. J. Physiol., 48, 340-71.

HYMAN, L. H., 1919b. "Physiological Studies on Planaria. I. Oxygen Consumption in Relation to Feeding and Starvation." Am. J. Physiol., 49, 377-402.

HYMAN, L. H., 1919c. "Physiological Studies on Planaria. II. Oxygen Consumption in Relation to Regeneration." Am. J. Physiol., 50, 67-81.

HYMAN, L. H., 1919d. "Physiological Studies on Planaria. III. Oxygen Consumption in Relation to Age (Size) Differences. Biol. Bull mar. biol. Lab., Woods Hole, 37, 388-403.

HYMAN, L. H., 1920. "Physiological Studies on Planaria. IV. A Further Study of Oxygen Consumption During Starvation." Am. J. Physiol., 53, 399-420.

HYMAN, L. H., 1923. "Physiological Studies on Planaria. V. Oxygen Consumption of Pieces with Respect to Length, Level, and Time After Section." J. exp. Zool, 37, 47-68.

HYMAN, L. H., 1924. "Some Technical Methods for Hydra and Planaria." Trans. Am. microsc. Soc., 43, 68-71.

HYMAN, L. H., 1941. "Environmental Control of Sexual Reproduction in a Planarian." Anat. Rec., 81, suppl., 108.

HYMAN, L. H., 1943. "Water Content of Medusae; Sexuality in a Planarian." Nature, Lond., 151, 140.

HYMAN, L. H., 1951. "The Invertebrates: Platyhelminthes and Rhynchocoela." Vol. II. pp.156-220. McGraw Hill, New York.

ISHII, S., 1966. "The Ultrastructure of the Insunk Epithelium Lining the Planarian Pharyngeal Cavity." J. Ultrastruct. Res., 14, 345-55.

JACOBSON, R., and JACOBSON, A., 1963. In Worms and Things (by J. V. McConnell), The Worm Runner's Digest, 5, No.1, p.2.

JENNINGS, J. B., 1957. "Studies on Feeding, Digestion, and Food Storage in Free-Living Flatworms (Platyhelminthes: Turbellaria)." Biol. Bull. mar. biol. Lab., Woods Hole, 112, 63-80.

KANATANI, H., 1958. "Formation of Bipolar Heads Induced by Demecolcine in the Planarian, Dugesia gonocephala." J. Fac. Sci. Tokyo Univ., Sec. 4, 8, 253-70.

KELLER, J., 1894. "Die Ungeschlechtliche Fortpflanzung der Süsswasser-Turbellarien." Jena Z. Naturw., 28, 370-407.

KEMBER, N. F., 1967. "Cell Survival and Radiation Damage in Growth Cartilage." Br. J. Radiol., 40, 496-505.

KENK, R., 1930. "Beiträge zum System der Probursalier (Tricladida paludicola)." Zool. Anz., 89, 145-62.

KOHL, D. M., and FLICKINGER, R. A., 1966. "The Role of DNA Synthesis in the Determination of Axial Polarity of Regenerating Planarians." Biol. Bull. mar. biol. Lab., Woods Hole, 131, 323-30.

KOLMAYER, S., and STÉPHAN-DUBOIS, F., 1960. "Néoblastes et Limitation du Pouvoir de Régénération Céphalique chez la Planaire Dendrocoelum lactuem."
J. Embryol. exp. Morph., 8, 376-86.

KNUDSEN, L. F., and CURTIS, J. M., 1947. "The Use of the Angular Transformation in Biological Assays."
J. Am. statist. Ass., 42, 282-96.

KRATOCHWIL, K. W., 1962. "Die Einwirkung von Röntgenstrahlen auf die Differenzierung in der Regeneration von Euplanaria gonocephala." Z. wiss. Zool., 167, 215-37.

LAJTHA, L. G., OLIVIER, R., and GURNEY, C. W., 1962. "Kinetic Model of a Bone-Marrow Stem-Cell Population."
Br. J. Haemat., 8, 442-60.

LAJTHA, L. G., and SUIT, H. D., 1955. "Uptake of Radioactive Iron (^{59}Fe) by Nucleated Red Cells in Vitro."
Br. J. Haemat., 1, 55-61.

LEA, D. E., 1955. "Actions of Radiations on Living Cells."
2nd ed., Cambridge University Press, Cambridge, England.

LEHNERT, G. H., 1891. "Beobachtung an Landplanarian." Arch. Naturgesch., 1, 306-50.

LE MOIGNE, A., 1962. "Étude de Formules Chromosomiques de Quelques Polycelis (Turbellariés, Triclades) de la Région Parisienne." Bull. Soc. zool. Fr., 87, 259-70.

LE MOIGNE, A., 1963. "Étude du Développement Embryonnaire de Polycelis nigra (Turbellarié, Triclade)." Bull. Soc. zool. Fr., 88, 403-22.

LE MOIGNE, A., 1965a. "Mise en Évidence d'un Pouvoir de Régénération chéz l'Embryon de Polycelis nigra (Turbellairié-Triclade)." Bull Soc. zool. Fr., 90, 355-61.

LE MOIGNE, A., 1965b. "Sur la Régénération et la Différentiation de Fragments d'Embryons de Polycelis nigra tenuis (Turbellarie Triclade)." C. r. Séanc. Soc. Biol., 159, 54-7.

LE MOIGNE, A., 1965c. "Effets des Irradiations aux Rayons X sur le Développement Embryonnaire et le Pouvoir de Régénération à l'Éclosion, de Polycelis nigra." C. r. hebd. Séanc. Acad. Sci., Paris, 260, 4627-9.

LE MOIGNE, A., 1966. "Étude du Développement Embryonnaire et Recherches sur les Cellules de Régénération chez l'Embryon de la Planaire Polycelis nigra (Turbellarié, Triclade)." J. Embryol. exp. Morph., 15, 39-60.

LE MOIGNE, A., SAUZIN, M. J., and LENDER, T., 1966. "Comparaison de l'Ultrastructure du Néoblaste et de la Cellule Embryo-

nnaire des Planaires d'eau Douce." C. r. hebd. Séanc. Acad. Sci., Paris, 263, 627-9.

LE MOIGNE, A., SAUZIN, M. J., LENDER, T., and DELAVAULT, R., 1966. "Quelques Aspects des Ultrastructures du Blastème de Régénération et des Tissus Voisins chez Dugesia gonocephala (Turbellarié, Triclade)." C. r. Séanc. Soc. Biol., 159, 530-8.

LENDER, Th., 1950. "Démonstration de Rôle Organisateur du Cerveau dans la Régénération des Yeux de la Planaire Polycelis nigra par la Méthode des Greffes." C. r. Séanc. Soc. Biol., 144, 1407-9.

LENDER, Th., 1952. "Le Rôle Inducteur du Cerveau dans la Régénération des Yeux d'une Planaire d'eau Douce." Bull. biol. Fr. Belg., 86, 140-215.

LENDER, Th., 1956a. "Recherches Expérimentales sur la Nature et les Propriétés de l'Inducteur de la Régénération des Yeux de la Planaire Polycelis nigra." J. Embryol. exp. Morph., 4, 196-216.

LENDER, Th., 1956b. "L'Inhibition de la Régénération du Cerveau des Planaires Polycelis nigra (EHRB.) et Dugesia lugubris (O. Schm.) en Présence de Broyats de Têtes ou de Queues." Bull. Soc. zool. Fr., 81, 192-9.

LENDER, Th., 1956c. "Analyse des Phénomènes d'Induction et d'Inhibition dans la Régénération des Planaires." Année biol., 32, 457-71.

LENDER, Th., 1960. L'Inhibition Spécifique de la Différentiation du Cerveau des Planaires d'eau Douce en Régénération." J. Embryol. exp. Morph., 8, 291-301.

LENDER, Th., and DEUTSCH, V., 1961. "L'Activité Inductrice du Broyat d'Embryon de Poulet au cours de la Régénération des Yeux de la Planaire Polycelis nigra." C. r. hebd. Séanc. Acad. Sci., Paris, 253, 550-1.

LENDER, Th., and GABRIEL, A., 1960. "Étude Histochimique des Néoblastes de Dugesia lugubris (Turbellarié, Triclade) Avant et Pendant la Régénération." Bull. Soc. zool. Fr., 85, 100-110.

LENDER, Th., and GABRIEL, A., 1961. "Le Comportement des Néoblastes Pendant la Regeneration de la Planaire Dugesia lugubris (Turbellarié, Triclade)." Bull. Soc. zool. Fr., 86, 67-72.

LENDER, Th., and GABRIEL, A., 1965. "Les Néoblastes Marqués par l'Uridine Tritiée Migrent et Édifient le Blastème de Régénération des Planaires d'eau Douce." C. r. hebd. Séanc. Acad. Sci., Paris, 260, 4095-7.

LENDER, Th., and KLEIN, N., 1961. "Mise en Évidence de Cellules Sécrétrices dans le Cerveau de la Planaire Polycelis nigra. Variation de leur Nombre au cours de la Régénération Postérieure." C. r. hebd. Seanc. Acad. Sci., Paris, 253, 331-3.

LENICQUE, P. M., and LUNDBLAD, M., 1966. "Promotors and Inhibitors of Development During Regeneration of the Hypostome and Tentacles of Hydra littoralis." Acta Zool., Stockh., 47, 277-88.

LEPORI, N. G., 1949a. "Ricerche sulla Ovogenesi e sulla Fecondazione nella Planaria Polycelis nigra Ehrenberg con Particolare Riguardo all'Ufficio della Nucleo Spermatico." Caryologia, 1, 280-95.

LEPORI, N. G., 1949b. "Il Ciclo Cromosomico, con Poliploidia, Endomitosi e Ginogenesi, in Popolazioni Italiane di Polycelis nigra Ehrenberg." Caryologia, 2, 301-24.

LINDH, N. O., 1959. "Heteroplastic Transplantation of Transverse Body Sections in Flatworms." Ark. Zool, 12, 183-95.

LØVTRUP, E., 1953. "Studies on Planarian Respiration." J. exp. Zool., 124, 427-34.

MAGANINI, G., 1961. "Cytological Researches on Populations of the Planarian Dugesia lugubris s. l. from North Ireland." Memorie Soc. tosc. Sci. nat., 68, Ser. B, 181-7.

MARSH, G., 1962. "Volume Resistivity of Regenerating Dugesia dorotocephala and the Electrical Work of Polarity Control." J. cell. comp. Physiol., 59, 273-80.

MARSH, G., and BEAMS, H. W., 1952. "Electrical Control of Morphogenesis in Regenerating Dugesia tigrina. I. Relation of Axial Polarity to Field Strength." J. cell. comp. Physiol., 39, 191-210.

McWHINNIE, M. A., 1955. "The Effect of Colchicine on Reconstitutional Development in Dugesia dorotocephala." Biol. Bull. mar. biol. Lab., Woods Hole, 108, 54-65.

MELANDER, Y., 1963. "Cytogenetic Aspects of Embryogenesis in Paludicola, Tricladida." Hereditas, 49, 119-66.

METALLI, P., and BALLARDIN, E., 1962. "First Results on X-ray-induced Genetic Damage in Artemia salina Leach." Atti Ass. genet. ital., 7, 219-31.

MORKOVIN, D., and FELDMAN, A., 1959. "Dosimetry in Living Cells Irradiated on Glass: A Correction." Br. J. Radiol., 32, 282-3.

MORTIMER, R. K., 1958. "Radiobiological and Genetic Studies on a Polyploid Series (Haploid to Hexaploid) of Saccharomyces cerevisiae." Radiat. Res., 9, 312-26.

NEEDHAM, A. E., 1952. "Regeneration and Wound-Healing." Methuen and Co. Ltd., London.

NIAS, A. H. W., GILBERT, C. W., LAJTHA, L. G., and LANGE, C. S., 1965. "Clone-Size Analysis in the Study of Cell Growth Following Single or During Continuous Irradiation." Int. J. Radiat. Biol., 9, 275-90.

OKADA, Y. K., and KIDO, T., 1943. "Further Experiments on Transplantation in Planaria." J. Fac. Sci. Tokyo Univ., IV, 6, 601-23.

OKADA, Y. K., and SUGINO, H., 1934. "Transplantation Experi-

ments in Planaria gonocephala I and II."
Proc. imp. Acad., Japan, 10, 37-40, 107-10.

OKADA, Y. K., and SUGINO, H., 1937. "Transplantation Experiments in Planaria gonocephala Dugès.
Jap. J. Zool., 7, 373-439.

OLIVER, R., 1964. "A Comparison of the Effects of Acute and Protracted Gamma-Radiation on the Growth of Seedlings of Vicia faba. Part II. Theoretical Calculations."
Int. J. Radiat. Biol., 8, 475-88.

OLMSTEAD, P. S., and TUKEY, J. W., 1947. "A Corner Test for Association." Ann. math. Statist., 18, 495-513.

PATERSON, E., 1942. "A Comparison of the Action of X and Gamma Radiation on Fibroblasts. Part II."
Br. J. Radiol., , 15, 302-6.

PEDERSEN, K. J., 1956. "On the Oxygen Consumption of Planaria vitta During Starvation, the Early Phase of Regeneration and Asexual Reproduction." J. exp. Zool, 131, 123-35.

PEDERSEN, K. J., 1958. "Morphogenetic Activities During Planarian Regeneration as Influenced by Triethylene Melamine." J. Embryol. exp. Morph., 6, 308-34.

PEDERSEN, K. J., 1959a. "Some Features of the fine Structure and Histochemistry of Planarian Subepidermal Gland Cells." Z. Zellforsch microsk. Anat., 50, 121-42.

PEDERSEN, K. J., 1959b. "Cytological Studies of the Planarian Neoblast." Z. Zellforsch microsk. Anat., 50, 799-817.

PEDERSEN, K. J., 1961a. "Studies on the Nature of Planarian Connective Tissue." Z. Zellforsch mikrosk. Anat., 53, 569-608.

PEDERSEN, K. J., 1961b. "Some Observations on the Fine Structure of Planarian Protonephridia and Gastrodermal Phagocytes." Z. Zellforsh mikrosk. Anat., 53, 609-28.

PETRUCCI, D., 1962. "Gradienti di Child, Respirazione ed. Ossidasi nelle Planarie." Ricerca scient., 32 (II-B), 79-96.

PIERAGNOLI, U., 1953. "Il Cariogramma di Dugesia lugubris (O. Schmidt) (Triclade paludicolo)." Memorie Soc. tosc. Sci. nat., 60, Ser. B, 31-5.

PIERAGNOLI, U., 1954. "Il ciclo Chromosomico in un Biotipo Poliploide de Dugesia lugubris (O. Schmidt) (Triclade Paludicolo)." Memorie Soc. tosc. Sci. nat., 61, Ser. B, 18-34.

POLLARD, E. C., and WHITMORE, G., 1955. "Deuteron Bombardment of Oriented Tobacco Mosaic Virus Preparations." Science, N.Y., 122, 335.

PORTER, E. H., 1964. "Electronic Computers and Survival Curves." Br. J. Radiol., 37, 610-5.

PORTER, E. H., 1967. "Personal Communication."

PRENANT, M., 1922. "Recherches sur le Parenchyme des Plathelminthes. Essais d'Histologie Comparée." Archs Morph. gén. exp., 5, 1-174.

PUCK, T. T., and MARCUS, P. I., 1956. "Action of X-rays on Mammalian Cells." J. exp. Med., 103, 653-66.

PUCK, T. T., MORKOVIN, D., MARCUS, P. I., and CIECIURA, S. J. 1957. "Action of X-rays on Mammalian Cells. II. Survival Curves of Cells from Normal Human Tissues." J. exp. Med., 106, 485-500.

RANDOLPH, H., 1897. "Observations and Experiments on Regeneration in Planarians." Wilhelm Roux Arch. Entw. Mech. Org., 5, 352-72.

RAVEN, C. P., and MIGHORST, J. C., 1948. "On the Influence of a Posterior Wound Surface on the Anterior Regeneration in Euplanaria lugubris (Hesse)." Proc. K. ned. Akad. Wet., 51, 434-45.

RÉVÉSZ, L., and LITTLRAND, B., 1964. "Variation of the Relative Sensitivity of Closely Related Neoplastic Cell Lines Irradiated in Culture in the Presence or Absence of Oxygen." Nature, Lond., 203, 742-4.

RÉVÉSZ, L., and NORMAN, U., 1960. "Chromosome Ploidy and Radiosensitivity of Tumours." Nature, Lond., 187, 861-2.

REYNOLDSON, T. B., 1960. "A Quantitative Study of the Population Biology of Polycelis tenuis (Ijima) (Turbellaria, Tricladida)." Oikos, 11, 125-41.

REYNOLDSON, T. B., 1961. "A Quantitative Study of the Population Biology of Dugesia lugubris (O. Schmidt) (Turbellaria, Tricladida)." Oikos, 12, 111-25.

RÖHLICH, P., 1966. "Sensitivity of Regenerating and Degenerating Planarian Photoreceptors to Osmium Fixation." Z. Zellforsh microsk. Anat., 73, 165-73.

ROSENBAUM, R. M., and ROLON, C. I., 1960. "Intracellular Digestion and Hydrolytic Enzymes in the Phagocytes of Planarians." Biol. Bull. mar. biol. Lab., Woods Hole, 118, 315-23.

ROWLEY, J., MULDAL, S., GILBERT, C. W., LAJTHA, L. G., LINDSTEN, J., FRACCARO, M., and KAIJSER, K., 1963. "Synthesis of Deoxyribonucleic Acid on X-Chromosomes of an XXXXY Male." Nature, Lond., 197, 251-2.

SANTOS, F. V., 1929. "Studies on Transplantation in Planaria." Biol. Bull. mar. biol. Lab., Woods Hole, 57, 188-97.

SANTOS, F. V., 1931. "Studies on Transplantation in Planaria dorotocephala and Planaria macculata." Physiol. Zool., 4, 111-64.

SCHAPER, A., 1904. "Experimentelle Untersuchungen über den Einfluss der Radiumstralen und der Radiumemanation auf Embryonale und Regenerative Entwicklungsvorgänge." Anat. Anz., 25, 298-314, 326-37.

SCHAROFF, J., 1934. "Über die Abhängigkeit des Regenerationsprozesses von Entwicklungsstadium und Alter bei Dendrocoelum lacteum (in Russian)." Trudy Lab. éksp. Zool. Morf. Zhivot., 3, 141-64.

SENGEL, Ph., 1951a. "Sur les Conditions de la Régénération Normale du Pharynx chez la Planaire d'eau Douce Dugesia lugubris O. Schm." Bull. biol. Fr. Belg., 85, 376-91.

SENGEL, Ph., 1951b. "Sur les Conditions de la Régénération Normale du Pharynx chez la Planaire Dugesia (= Euplanaria) lugubris O. Schm." C. r. Séanc. Soc. Biol., 145, 1381-4.

SENGEL, Ph., 1953. "Sur l'Induction d'une Zone Pharyngienne chez la Planaire d'eau Douce Dugesia lugubris O. Schm." Archs Anat. microsc., 42, 57-66.

SERLIN, I., and COTZIAS, G. C., 1957. "State of Tissue Acetylcholinesterase as Determined by Cobalt-60 Gamma Radiation Inactivation." Radiat. Res., 6, 55-66.

SHAPIRA, J., COLEMAN, J. O., and CASTELLANI, P. C. F., 1966. "Differential Nucleic Acid Metabolism of Planarial Segments." Proc. Soc. exp. Biol. Med., 122, 1266-9.

SILINI, G., and HORNSEY, S., 1962. "Studies on Cell-Survival of Irradiated Ehrlich Ascites Tumour. III. A Comparison of the X-ray Survival Curves Obtained with a Diploid and a Tetraploid Strain." Int. J. Radiat. Biol., 5, 147-153.

SINCLAIR, W. K., and MORTON, R. A., 1963. "Variations in X-ray Response During the Division Cycle of Partially Synchronized Chinese Hamster Cells in Culture." Nature, Lond., 199, 1158-60.

SINGER, M., 1952. "The Influence of the Nerve in Regeneration of the Amphibian Extremity." Q. Rev. Biol., 27, 169-200.

SIVICKIS, P. B., 1932. "A Quantitative Study of Regeneration along the Main Axis of the Triclad Body." Archo. zool. ital., 16, 430-49.

SIVICKIS, P. B., 1933. "Studies on the Physiology of Regeneration in Triclads." Vytauto Didžiojo Univ. Mat.-Gamtos Fak. Darbai (Mém. Fac. Sci. Vyautas le Grand) /Kaunas/, 1, 369-441.

SIVICKIS, P. B., 1934. "The Effect of Regeneration Activity upon the Rate of Regeneration of Eyes in Planaria lugubris." Vytauto Didžiojo Univ. Mat.-Gamtos Fak. Darbai (Mém. Fac. Sci. Vyautas le Grand) /Kaunas/, 2, 13-42. Abstract No. 13196, Biol. Abstr., 10.

SKAER, R. J., 1961. "Some Aspects of the Cytology of Polycelis nigra." Q. Jl. microsc. Sci., 102, 295-318.

SKAER, R. J., 1965. "The Origin and Continuous Replacement of Epidermal Cells in the Planarian Polycelis tenuis (Iijima)." J. Embryol. exp. Morph., 13, 129-39.

SPARROW, A. H., 1962. "The Role of the Cell Nucleus in Determining Radiosensitivity." Brookhaven Lecture Series No.17 (BNL Y.)."

SPARROW, A. H., 1963. In Discussion of Paper Raporteured by H. J. Evans in Radiation Effects in Physics, Chemistry and Biology, ed. Ebert, M., and Howard, A., North-Holland Publishing Co., Amsterdam.

SPARROW, A. H., and EVANS, H. J., 1961. "Nuclear Factors Affecting Radiosensitivity. I. The Influence of Nuclear Size and Structure, Chromosome Complement, and DNA Content." Brookhaven Symp. Biol., 14, 76-100.

SPARROW, A. H., and MIKSCHE, J. P., 1961. "Correlation of Nuclear Volume and DNA Content with Higher Plant Tolerance to Chronic Radiation." Science, N.Y., 134, 282-3.

SPARROW, A. H., SPARROW, R.C., SHAIRER, L. A., and POND, V., 1965. "Evidence that Genetic Redundancy Does not Have a Radioprotective Effect in Polyploids of Higher Plants." Radiat. Res., 25, 243, Abstr. No. 194.

STEINMANN, P., 1908. "Untersuchungen über das Verhalten der Verdauungssystems bei der Regeneration der Tricladen." Wilhelm Roux Arch. Entw. Mech. Org., 25, 523-68.

STEINMANN, P., and BRESSLAU, E., 1913. "Die Strudelwurmer (Turbellaria)." Monograph. einheimischer Tiere 5, Leipzig.

STÉPHAN-DUBOIS, F., 1951. "Migrations et Potentialités Histogénétiques des Cellules Indifférenciées chez les Hydres, les Planaires et les Oligochètes." Annls. Biol., 27, 734-50.

STÉPHAN-DUBOIS, F., 1961. "Les Cellules de Régénération chez la Planaire Dendrocoelum lacteum." Bull. Soc. zool. Fr.,

86, 172-185.

STÉPHAN-DUBOIS, F., 1965. "Les Néoblastes dans la Régénération chez les Planaires" in Regeneration in Animals and Related Problems, ed. V. Kiortsis and H. A. L. Trampusch, North-Holland Publishing Co., Amsterdam.

STÉPHAN-DUBOIS, F., 1966. "Personal Communication."

STÉPHAN-DUBOIS, F., and GILGENKRANTZ, F., 1960. "Régénération après Transplantation chez la Planaire Dendrocoelum lacteum." C. r. Séanc. Soc. Biol., 155, 115-8.

STÉPHAN-DUBOIS, F., and KOLMAYER, S., 1959. "La Migration et la Différentiation des Cellules de Régénération chez la Planaire Dendrocoelum lacteum." C. r. Seanc. Soc. Biol., 153, 1856-8.

STOLTE, H. A., 1936. "Die Herunft der Zell Materials bei Regenerativen Vorgängen der Wirbellosen Tierre." Biol. Rev., 11, 1-48.

STRANDSKOV, H. H., 1937. "Certain Physiological Effects of X-rays on Euplanaria dorotocephala. II. Regeneration." Physiol. Zool., 10, 14-20.

SUGINO, H., 1940. "Influence of the Head Pieces Implanted in the Prepharyngeal Region of Planarian." Ann. Zool. Jap., 19, 245-52.

SUGINO, H., 1941. "Homopolar Union in Planaria gonocephala." Jap. J. Zool., 9, 175-83.

TERASIMA, T., and TOLMACH, L. J., 1961. "Changes in X-ray Sensitivity of Hela Cells During the Division Cycle." Nature, Lond., 190, 1210-11.

TESHIROGI, W., and JIN, A., 1964. "The Relation Between Regeneration and the Nervous System in the Freshwater Planarian Bdellocephala brunnea. I. Regeneration by a cut Piece Lacking the Ventral Nerve Cords." Zool. Mag., Tokyo, 73, 45-51.

TILL, J. E., 1961. "Radiosensitivity and Chromosome Numbers in Strain L Mouse Cells in Tissue Culture." Radiat. Res., 15, 400-9.

TILL, J. E., and McCULLOCH, E. A., 1961. "A Direct Measurement of the Radiation Sensitivity of Normal Mouse Bone Marrow Cells." Radiat. Res., 14, 213-22.

URBAN, F. M., 1910. "Die Psychophysischen Massmethoden als Grundlagen Empirischer Messungen." Arch. ges. Psychol., 16, 168-227.

VANDEL, A., 1920. "Le Développement de l'Appareil Copulateur des Planaires est sous la Dépendence des Glandes Genitales." C. r. hebd. Séanc. Acad. Sci., Paris, 170, 249-54.

VANDEL, A., 1921. "La Régénération des Glandes Génitales chez les Planaires." C. r. hebd. Séanc. Acad. Sci., Paris, 172, 1072-4.

VAN 'T HOF, J., and SPARROW, A. H., 1965. "Radiation Effects on the Growth Rate and Cell Population Kinetics of Actively Growing and Dormant Roots of Tradescantia paludosa." J. Cell Biol., 26, 187-99.

VERHOEF, A. M., 1946. "The Mitotic Activity during the Regeneration of Polycelis nigra." Proc. K. ned. Akad. Wet., 49, 548-53.

WAGNER, F. VON, 1890. "Zur Kenntnis der Ungeschlechtlichen Fortpflanzung von Microstoma Nebst Allgameinen Bemerkungen über Teilung und Knosprung im Tierreich." Zool. Jb., 4, 349-423.

WATANABE, Y., 1935a. "Rate of Head Development as Indicated by Time of Appearance of Eyes in the Reconstitution of Euplanaria dorotocephala." Physiol. Zoöl., 8, 41-64.

WATANABE, Y., 1935b. "Head Frequenty in Euplanaria maculata in Relation to the Nervous System." Physiol. Zoöl., 8, 374-94.

WEIGAND, K., 1930. "Regeneration bei Planarien und Clavelina unter dem Einfluss von Radium-Strahlen." Z. wiss. Zool., 136, 255-318.

WILSON, J. W., 1941. "The Regeneration of the Planarian Head in Diluted Ringer's Fluid." J. exp. Zool., 86, 225-46.

WITHERS, H. R., 1967. "The Dose- Survival Relationship for Irradiation of Epithelial Cells of Mouse Skin." Br. J. Radiol., 40, 187-94.

WOLFF, Et., and DUBOIS, F., 1947a. "La Migration des Cellules de Régénération et les Facteurs qui la Provoquent chez les Triclades." C. r. hebd. Séanc. Acad. Sci., Paris, 224, 1387-8.

WOLFF, Et., and DUBOIS, F., 1947b. "Sur une Méthode d'Irradiation Localisée Permettant de Mettre en Évidence la Migration des Cellules de Régénération chez les Planaires." C. r. Séanc. Soc. Biol., 141, 903-6.

WOLFF, Et., and DUBOIS, F., 1947c. "Sur les Facteurs qui Déclenchent la Migration des Cellules de Régénération chez les Planaires." C. r. Séanc. Soc. Biol., 141, 906-9.

WOLFF, Et., and DUBOIS, F., 1948a. "Sur la Migration des Cellules de Régénération chez les Planaires." Revue suisse Zool., 55, 218-27.

WOLFF, Et., and Dubois, F., 1948b. "Mise en Évidence d'un Système de Corrélations Intervenant dans la Régénération des Planaires d'eau Douce." Experientia, 4, 273-5.

WOLFF, Et., and LENDER, Th., 1950. "Sur le Determinisme de la Régénération des Yeux chez une Planaire d'eau Douce Polycelis nigra." C. r. Séanc. Soc. Biol., 144, 1213-6.

WOODRUFF, L. S., and BURNETT, A., 1965. "The Origin of the Blastemal Cells in Dugesia tigrina." Expl. Cell Res., 38, 295-305.

ZELENY, Ch., 1917. "The Effect of Degree of Injury, Level of Cut and Time Within the Regeneration Cycle upon the Rate of Regeneration." Proc. natn. Acad. Sci. U.S.A. 3, 211-17.

ZIRKLE, R. E., 1957. "Partial-Cell Irradiation." Advances in Biological and Medical Physics, volume V, Academic Press Inc., New York.

ZIRKLE, R. E., and TOBIAS, C. A., 1953. "Effects of Ploidy and Linear Energy Transfer on Radiobiological Survival Curves." Archs Biochem. Biophys., 47, 282-306.

OTHER LITERATURE CITED

HALL, E. J., 1962. "A Method of Deducing a Dose-Response Relationship for Reproductive Integrity of Cells Exposed to Radiation by Means of Fractionation Experiments." Br. J. Radiol., 35, 398-402.

HORNSEY, S., 1967. "The Recovery Process in Organized Tissue", in Radiation Research, ed. G. Silini, North-Holland Publishing Co., Amsterdam.

WILSON, B. R., 1963. Radiat. Res., 20, 477. /Cited by Hornsey (1967)/.