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GRADIENTS OF VITAL STAINING AND SUSCEPTIBILITY IN PLANARIA AND OTHER FORMS

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DEPARTMENT OF ZOÖLOGY

BY

JOHN WOOD MAC ARTHUR

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JOHN WOOD MACARTHUR

From the Department of Zoölogy, University of Chicago

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The investigations described in this series were undertaken in an attempt to reveal more facts as to the nature of the metabolic factors controlling individual organization and development, and to assign to these factors their proper relative values. Since recent work along this line rests largely upon a definite background of previous work, particularly that of Child, some of the conclusions of this writer may be summarily reviewed, with the caution that no statement or interpretation should be attributed to him without first consulting his own writings (1), (2), (3).

After extended experimentation and careful analysis of phenomena of regulation, growth and development in many organisms, Child put forth certain helpful generalizations as to the dynamic nature of the organism, which were applied convincingly to most varied and apparently independent groups of data. According to this view it is held that: metabolism is the basis of the phenomena of life, and an axiate "organic individual in its simplest terms" consists of a quantitative "metabolic gradient, or gradients in certain metabolic reactions, perhaps oxidations, with associated protoplasmic conditions," existing along the main axis and probably also in minor axes; the establishment of such a physiological gradient or gradients by interaction of environment and specific protoplasm is the first prerequisite to development and organization, and constitutes the basis of the functional and structural symmetry and polarity of the individual; through transmission of excitations the region of highest metabolic activity in the axial gradient exerts a dominating and integrating influence over subordinate levels with a lesser metabolic rate, such dominance or control being manifested by a correlating, coördinating, and generally unifying action in ontogeny, growth, regulation, behavior, etc.

Proofs of the existence of such a metabolic gradient and evidence of its nature may be found in the literature cited. These proofs and evidences have hitherto concerned themselves chiefly with differences along the axis in regulation capacity, in susceptibility, in output of CO_2, in consumption of O_2, and in electrical potential. Numerous other differences, often closely associated with metabolic activity, might well be sought for and studied in favorable forms, e.g., differences in heat production, in electrical conductivity, in H ion concentration, in water content, in permeability of membranes, in state of dispersal of colloids, etc. The rôle of each of these factors deserves individual attention, especially because of the wide applicability of the results in physiological gradients and in metabolism generally.

The writer believed that an attack of the problem might be made by a study of the action of electrolytes and dyes. Certain aspects of H ion action have been treated (4), and results with salts will be reported later. The object of the present paper is chiefly to state the experimental facts as observed regarding gradients of staining and susceptibility in several flatworms, protozoa, hydra, annelids, and the chick embryo with vital and other dyes; to analyze and interpret as far as possible these results in their bearing upon the concept of metabolic gradients as a further test of its validity and applicability; and, specifically, *to ascertain whether regions of high general susceptibility and rapid respiratory exchange behave in a characteristic manner in the staining process* as shown by the diffusion, segregation, flocculation, etc., of the dyes. The results are believed to contribute additional proof, with agents hitherto little employed, of the reality of the metabolic gradient in the forms used, and further evidence as to the nature of these gradients, particularly with regard to certain physico-chemical properties associated with high metabolic activity.

The experiments were performed for the most part at the University of Chicago in 1916-1917; the paper was then put in substantially its present form. Now newer observations and more recent literature are included. While the writer naturally assumes full responsibility for the results embodied in this work, he gratefully acknowledges his debt throughout to Dr. C. M. Child for many kindnesses and for the unfailing suggestiveness of his writings and criticisms.

GENERAL STATEMENT OF RESULTS

As might perhaps have been anticipated, there is a rather clean-cut difference between basic and acid dyes in their staining capacities

intra vitam, corresponding to the known differences in their physical and chemical properties. Basic dyes alone truly and definitely stain the tissues of the organisms under observation; though acid dyes sometimes penetrate and are even stored in granules, they do not in general become visible by fixed staining of protoplasm. Basic dyes of most varied chemical constitution and relationship were used but, while they differed very considerably in toxicity, in irritating action and in details of staining, the final staining pictures obtained with all were essentially similar. Most basic dyes, as toluidin blue, Victoria blue, crystal violet, methylene blue, janus green, etc., and even neutral red, are much more toxic than the acid dyes, as congo red, eosin, erythrosin, trypan blue, methyl orange, acid fuchsin, orange G, etc.

A given tissue or layer does not stain uniformly and simultaneously throughout the length of the specimen, even though at final saturation just before death the intensity of stain may become approximately equal everywhere. Regions of strongly marked susceptibility to such lethal agents as had been used and to acids, alkalies, and the dyes themselves, are regions where the basic dyes first became detectable in certain granules or globules of the cells. Thus in general a staining gradient is produced indicating directly the metabolic gradient. A gradation of penetration was found with every major gradation of susceptibility.

Depth of coloration increases rapidly as the death point is neared, but preliminary to the actual onset of disintegration there occurs in most species with most basic dyes a sudden loss of both natural pigment materials and stained particles, leaving the most susceptible parts strikingly decolorized.

In causing a selective disintegration methylene blue and some other dyes proved to be favorable agents for demonstrating not only the chief longitudinal axis in flatworms but also in many cases the minor axes as well. But these axes were not always to be distinguished by differences in staining, nor is there noticable difference in rate of staining of young and old individuals.

EXPERIMENTAL

Methods. Dye samples were obtained from as varied sources as possible (Grubler's, also Bausch and Lomb's and Kahlbaum's). Stock solutions were made up in well or tap—rarely in distilled—water, and were not made free from the salt impurities with which they were dispensed.

In vital staining it is highly important that the experimental animals be healthy and that they be kept under almost continuous observation to

watch the progress of the staining since very erroneous notions may be obtained by examining the specimen at the end of the process when tissues have been loaded to saturation. The staining should be witnessed as a process rather than observed in the finished state. Animals were usually brought under observation in clear water and closely examined from time to time with eye, lens, dissecting or compound microscope, and were often flattened under a cover slip with or without support, or teased.

The salient features of *the susceptibility method* have been many times described. According to the concentration of chemical agents or the intensities of physical conditions used there are two general modes of studying relative susceptibility, the direct and the indirect, both of which seem to have a definite and characteristic relation to the metabolic rate. With the *direct* method such concentrations or intensities are used as are lethal within a few hours; in this case individuals or parts with highest metabolic rate are most susceptible, and the susceptibility gradient follows the metabolic gradient. With the *indirect* method such lower concentrations or intensities are used that some acclimation occurs and death takes place only after many hours or days; in this case acclimation is most rapid and complete in individuals or parts with highest metabolic rate and length of life varies directly with rate of reaction.

As *criteria of* relative *susceptibility* use was made of loss of motility or response to stimulation, possibility of recovery, etc. For most lower invertebrates disintegration is a satisfactory index, and appropriate for fairly exact and quantitative readings. Death is manifested by a loss of continuity of surface contour, swelling, and loss of constituents, by the visible separation of tissue fragments and cells from the main mass, and by change to acid phase of some dye indicators (e.g., neutral red). With the usual slight disturbances in the container tissue fragments continue to scatter out into the medium until little or nothing remains *in situ* of the dead parts. A disintegrating organism thus imparts a turbidity and a tinge of color to clear water. Impending disintegration is often indicated by cloudy swelling, opacity, immobility and loss of local responses. Accompanying color changes consist of loss of more or less natural pigment or previously stored stain. Criteria of equivalent staining rates are those of direct observation and readings on the time required for first visible coloration of a given part.

Factors modifying the effect of dyes: Concentration. Figure 1, summarizing averaged data collected from many protocols, shows the

fairly direct relation existing between concentration and time of first staining, staining at all levels, and first disintegration of *Planaria dorotocephala;* vital staining is roughly a function of time and concentration. The interval elapsing between a given intensity of staining and the initial disintegration widens with increasing dilution, until the

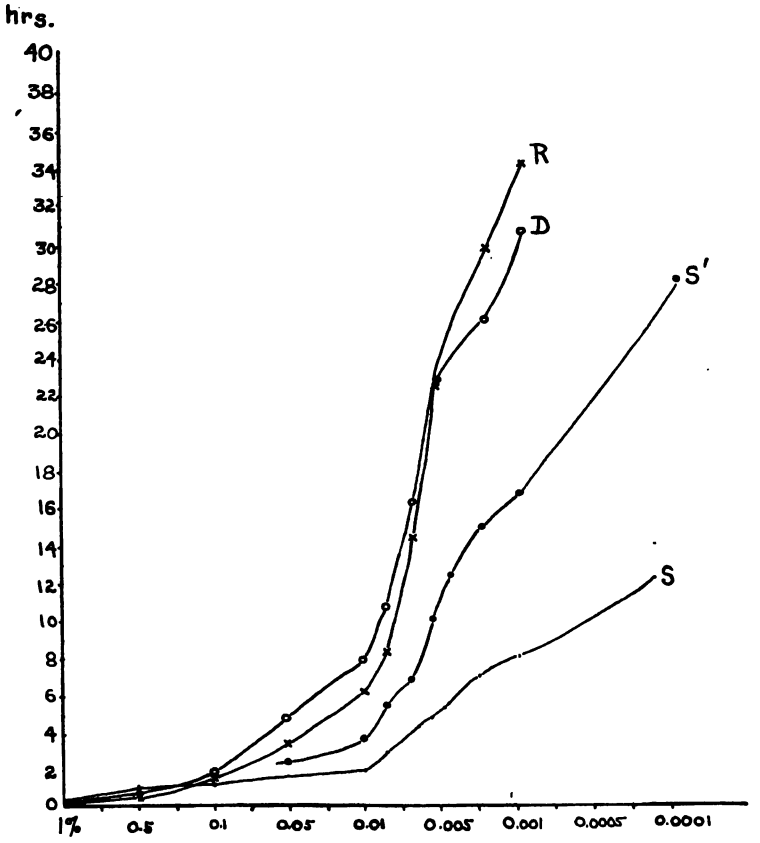

Fig. 1. Times of first visible staining, *S*, of first visible staining at all levels, *S'*, of first disintegration, *D*, and of longest possible exposure with complete or partial recovery, *R*, of *Planaria dorotocephala* of about 15–18 mm. length in different per cent concentrations of methylene blue at room temperature.

animals, though ultimately well stained, disintegrate less and less completely with greater individual variations, and finally near 0.0001 per cent will live on stained for an indefinite period either wholly intact or after recovery with loss of head and other most susceptible parts. In less toxic dyes like neutral red or even dilute methylene blue the worms

survive for months, carrying the dye even past fission and regeneration crises. In 0.1 per cent methylene blue loss of head substance occurs before stain has become at all visible in caudal regions, and in slightly higher concentrations toxic effects may be produced without appreciable visible staining, for recovery is impossible when staining begins. In short the curves of staining, S, S', do not run parallel with that of recovery, R, or that of initial disintegration, D. With a given intensity of stain prognosis for recovery is least favorable from strong and practically certain from weak solutions. Disintegration and failure to recover apparently depend less upon actual staining than upon presence of excess stain in the medium.

So far in this work the reversed order of susceptibility (indirect) has not been met with even in most dilute solution. But mere traces of methylene blue are said to have an accelerating effect upon growth of yeast.

Age of the organism is doubtless a factor in determining dye action. Ten large and ten small planarians were immersed together in 0.1 per cent methylene blue and examined at half-hour intervals and records made of the progress of their disintegration. A graph of the results shows a little difference in the resistance of the two sets of animals, the younger ones being perhaps slightly more susceptible than the larger. Similar results are obtained with acids (4).

Temperature. At 14°C. staining in 0.02 per cent m.b. is strikingly delayed as compared with that under the same conditions at 23°C. The protective effect of cold on organisms in the dye is so marked and the rates of staining and disintegration are so similarly modified that the clue might be followed further for evidence on the exact value of the temperature coefficients for intake of dye and for disintegration—particularly since amount of adsorption has a negative temperature coefficient (5). It would seem that adsorption is soon followed by chemical combination.

Hydrogen ion concentration. The reaction of a dye solution is of prime importance. Staining in well or lake water of pH = 7.5 may be considered rapid; if this water be made more alkaline by the addition of NaOH rate and depth of staining increases with rise of pH. At the same time in the more alkaline media differences in sites of staining also appear in that certain irregular or stellate bodies with nucleus-like centers stain conspicuously on the ventral surface, and in that blue granules are detectable in the posterior zoöid region of *P. dorotocephala* very shortly after similar ones are visible anteriorly and before any are

to be seen at intermediate levels. As OH' is reduced by addition of HCl the staining becomes more and more limited to the auricles and tip of the head ($pH = 6.4$), and finally at $pH = 4.8$ stained tissue cannot be found anywhere even after hours of exposure, when the acid itself kills. Probably for this reason animals stained very poorly in distilled water solutions of the dyes, for its reaction was about $pH = 6.0$. As a rule this species dies from the toxic effects of the distilled water before more than the few most sensitive parts of the head have been stained. In fact it is extremely difficult to get any basic stain at all into some specimens of oligochetes and protozoa when taken from an old, very acid culture. Sufficiently acid media apparently reversibly destain some vitally stained protoplasms to a certain extent; in many cases m.b. passes through a green or a more or less decolorized state. How much all these results are due to influence of the H ion concentration on dissociation and rate of diffusion of the dyes and how much to the alteration of membranes and deeper tissues is yet undetermined.

Susceptibility of all parts to a basic dye is usually increased by a definite alkaline reaction in the medium. In planaria the disintegration of the head is followed at once by the disintegration of the posterior zoöid region—an order which is not often followed if the reaction be acid. Alkalies seem to sensitize certain parts of the organism so that non-lethal concentrations of either the alkali or the basic dye combine to become lethal. It has been reported that alkalies increase direct susceptibility and acids indirect susceptibility (1), (4). But this fact should not be confused with another, namely, that an *inner* acid reaction probably increases suceptibility to basic dyes (6); both of these facts are consistently interpreted in the discussion.

Neutral salts markedly retard or actually prevent staining of all parts of planarians with m.b and other basic dyes. In this, $CaCl_2$ is more effective than NaCl. It is surely significant, however, that salts and hydrogen ion facilitate acid dye action.

Data in detail. The bulk of the data deals with the effect of m.b. on diverse species, and the results obtained are described for this dye with each form, frequent notes being added for other basic and a few acid dyes, when peculiarities or divergences in action were observed.

Planaria dorotocephala. This flatworm was singled out for particular study with the dyes because it was readily available and especially because it had been extensively used in the same laboratory for similar studies, and many data were already at hand of much value for comparison. Previous work with this had shown that individuals over

5 mm. are composed of a large anterior zoöid and of one or more smaller posterior zoöids not morphologically differentiated but clearly distinguishable physiologically (1). With these in mind attention was devoted chiefly to the relative speed and intensity of staining of different levels along the axis and to the time of disintegration of these different levels.

Staining gradient. Immersed in a solution of basic dye, planarians do not stain uniformly and simultaneously even throughout their surface area. Certain points of election are from the first visible. In general

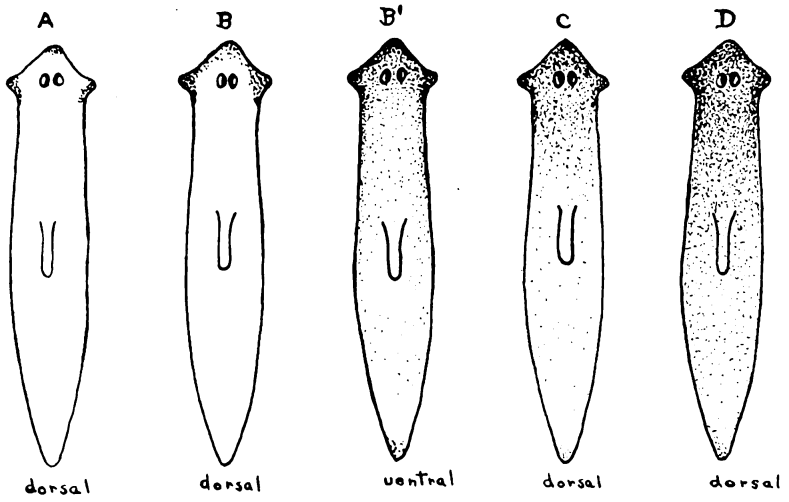

Fig. 2. Stages of staining of *P. dorotocephala* with methyene blue (and many other basic dyes) to show sites and relative intensity of coloration: *A*, stain in sensory lobes chiefly; *B*, spreading along margins of the head, and *B'*, same stage in ventral view, stain extending much farther than in *B*; *C*, and *D*, continued deep staining in more posterior levels. In alkaline media or with very young worms the stain shows early also in the posterior tip.

the first staining occurs within a few minutes or hours in certain parts of the epidermis. This order of such staining (fig. 2) is, practically without exception, first the lateral auricles, *A*, then the tip and later the ventral surface and margins of the head, *B*, *C*, continuing posteriorly thence until the whole animal excepting the proboscis is distinctly bluish, *D*, and finally quite blue-black externally. By use of diluter solutions and removal of specimens to clear water from time to time at appropriate intervals a series is obtained showing stained areas extending progressively backward and intensifying. At an early stage the animal is

quite distinctly "cyanocephalous" and is much given to moving the head in constant exploratory movement, or holding it stiffly erect. The ventral surface of the head takes on and retains a deeper coloration than the caudal parts and the dorsal surface, this difference persisting through further staining until shortly before cytolysis begins. In concentrated solutions the staining becomes more equalized throughout, and the initial differences are less evident. Curiously the proboscis remains strikingly uncolored even to the last in many dyes unless treated in a special manner to induce staining. In later stages of deep staining the anterior end is rendered more and more inactive, flaccid and unresponsive, and the part previously held erect drops under the ventral surface and the remaining parts roll or coil dorsally at ends and at the margins, in a characteristic fashion. If stimulated the animals may yet unroll and glide stiffly with the posterior cilia or muscles, but the uplifted anterior end is not touched to the substratum.

Teasing and close examination show the dye to be located in certain stained droplets and granules as well as to a less extent in the ground substance, first of the superficial cells and later in the deep-lying tissue. As a rule the stained constituents prove to be chiefly globules of an ever increasing size and number, occurring singly or several together inside larger globules of a bluish liquid. In m.b. made strongly alkaline the stain appears to pick out and color a number of irregular cells with central nuclei (?) situated on the ventral surface; no attempt was made to localize this stain by study of prepared sections.

Repeated tests made in various ways by exposing to H_2O_2 planarians stained only cephalically gave no indication of there being any colorless leucobase present but invisible in unstained regions, where it might conceivably be reduced. In fact, as Ehrlich found for m.b. in nervous tissue (7), the expectation would be that regions of high oxidative metabolism would reduce dye compounds to a leucoform more rapidly than the less active parts here left unstained.

Injured loci, wherever situated, take up basic stains considerably in advance of any uninjured parts: fission planes, either freshly or recently exposed or after the ends have contracted down and begun healing and reconstitution, exhibit a similar precocious coloration. There is no observational evidence for believing that simple exposure of or removal of a membrance from interior substances will promote immediate staining; the increased staining is such as would be expected to proceed from the stimulation of injury or the higher metabolism of contracting ends. It is interesting also to note that regenerating heads,

however translucent and apparently devoid of "density" and of differentiated structural material, yet stain easily and relatively deeply.

Previous killing by slow heating or by alcohol allows stains to flood in rapidly at all levels. Only in life was the staining gradient with basic dyes obtained.

Disintegration gradient. As bilaterally symmetric animals, flatworms possess three axes: the chief, antero-posterior axis, a ventro-dorsal one, and a medio-lateral one in the horizontal plane. Each of these axes should theoretically be represented by a gradient in metabolic or protoplasmic condition, such that a region of highest rate might be distinguished by its more marked susceptibility from other regions of lower rate.

As has been stated, there are low concentrations of stains which will stain without producing lethal effects anywhere. Once this minimal concentration is passed it is only a question of time when disintegration will set in. After the necessary toxic effect has been produced, the epidermal cells of the anterior end along the auricles and tip and ventral surface of the margins of the head assume a swollen and edematous aspect, loosen up from each other, lose their coherence and their original structural orientation, and scatter in small shreds, clumps and spherical masses, usually as small as the globules or granules composing the protoplasm. A disintegrating area decolorizes somewhat by extrusion of the stained constituents, so that the disintegrating portion is often sharply contrasted with the intact blue portion, as a loose, white, felt-like, downy tuft.

The order of disintegration of the epidermis and body wall (fig. 3) is most significant, since it affords an excellent readily visible demonstration of all of the three gradients believed to be present in the outer layers of the triaxiate organism. *a.* The zone of decoloration and of disintegration begins invariably on the auricles and tip and margins of the head, and proceeds slowly caudad. The disintegration belt is constantly shortened in front by detachment and loss of granules and droplets, and lengthened behind by the incorporation of more sound tissue into the disintegrating zone, which finally reaches the extreme posterior end. The belt immediately behind the disintegrating level is strongly contracted as in the sphincter-like closing of any injured part. Meanwhile the attitude of the head, margin and body is as described above. Parts left intact usually will exhibit some movements upon stimulation; only the more posterior levels retain power of adhering to the substratum. For a few minutes preceding initial

disintegration the individual passes through a stage of rhythmic movements; the wave proceeds postero-anteriorly—the posterior tip extends to its maximum caudally and then widens and shortens from the caudal tip forward, as if in attempting backward movement of an avoiding reaction. In moderate concentrations these rhythms may reverse in direction, several times alternating from posterior-anterior to anterior-posterior. *b*. The ventro-dorsal axis is also clearly indicated. Whitening and dissolution of tissue usually extend caudad more rapidly on the

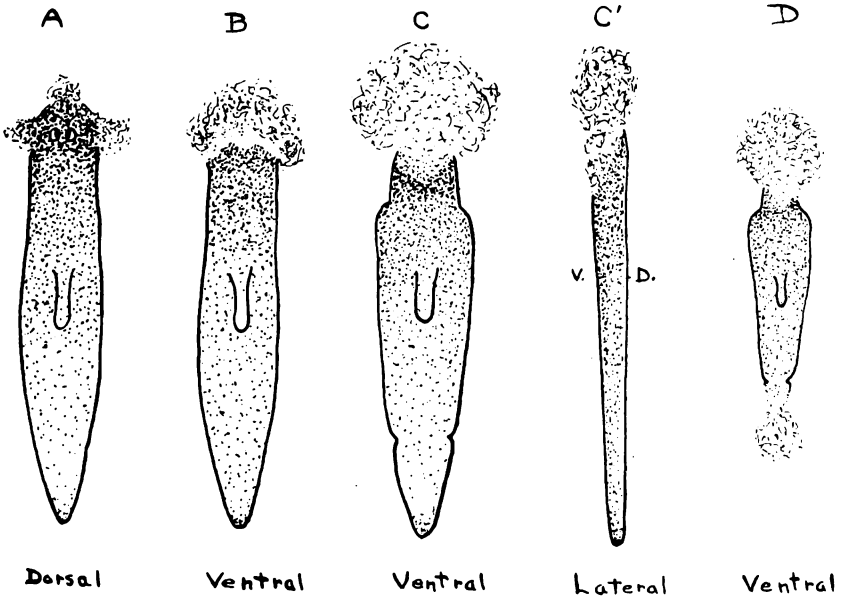

Fig. 3. Certain stages of disintegration of *P. dorotocephala* in methylene blue, showing correspondence with stages of staining of figure 2; and also the frequent precedence of ventral over dorsal disintegration, *C'*, and of median over lateral disintegration, *C*; *D*, and *D* the posterior disintegration of younger worms, or of any worm in a definitely alkaline medium of the dye.

ventral surface than on the dorsal (fig. 3, *C*). This more rapid advance of disintegration ventrally is obvious from the time the first tissue is lost under the head and is still noticeable posteriorly; in fact in many cases the ventral surface has entirely disintegrated when the dorsal parts of the posterior end are still intact in the form of a carapace-like shield of tissue, which is the last to display irritability and to disappear. *c*. Even the median-lateral axis is often demonstrable. Disintegration of the ventral surface does not usually advance back-

ward on an even transverse front. Often, and especially in high H ion concentration, disintegration of the epithelium is most rapid in the midline, so that the decolorized and cytolyzed area pushes back in a wedge shape and the living tissue is cut out to show a V-shaped front with two lateral arms projecting forward. In dorsal view the picture is more variable, and toward the posterior end the line of disintegration becomes more transverse.

In younger animals the posterior tip commonly disintegrates soon after the head. In more alkaline media all animals disintegrate thus (fig. 3, *D*). In no case was there any early loss of tissue at the anterior end of the second zoöid with basic dyes. A recent fission plane shows early disintegration corresponding to its early deep staining.

With dilute cyanide and various anesthetics Child obtained first disintegration along the margins, at the posterior tip, and dorsally at the anterior ends of intermediate zoöids. These results could not be duplicated with any basic dye or with acids, but were approached closely by use of alkalies and some acid dyes, as alizarin blue S (fig. 4).

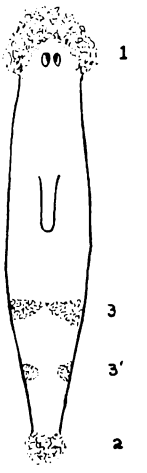

Fig. 4. An early stage of the disintegration of *P. dorotocephala* in alizarin blue S, and acid dye. Large specimens commonly show disintegration in the order *1, 2, 3, 3'*, or *1, 2, 3', 3.*

Other basic dyes differ from methylene blue chiefly in the degree of their irritating and toxic effects and in minor details of their staining. Naturally those with colors differing widely from the natural yellow brown pigment are most favorable for study of penetration of the dye, but all lend themselves to use as agents in susceptibility work—neutral red, crystal violet, victoria blue, magenta red, janus green, toluidin blue. The last two perhaps best show early staining of the posterior zoöid region.

Acid dyes—eosin, erythrosin, trypan blue, methyl blue, water blue, berlin blue, acid fuchsin, congo red, and many others—were tried in neutral solution but none became visible within the living animal even after hours or days. Only a few were toxic enough to kill. After death the dyes passed in but were easily washed out again. The action of alizarin blue S has been mentioned above. In more acid media the acid dyes are more effective, but here the acid effects seem to be predominant.

Planaria velata. In all essential respects this species resembles the above. Basic dyes penetrate and become visible within the proto-

plasm first in the truncate end and anterior margins of the head, and only later are to be seen at more posterior levels. *P. maculata* is even more similar to *dorotocephala*.

Phaenocora agassizi. This small white transparent rhabdocoel with large cilia was first met with and collected in abundance preying in a thriving ameba culture. On account of its small size (4–5 mm.) all observations were made under the microscope. This form is of some interest, for an individual is composed of a single zoöid and shows a simple steep main gradient. This gradient may be demonstrated with basic dyes in several distinct ways:

1. The staining gradient. Placed in 0.01 per cent m.b. the animal takes up the stain in a clearly differential fashion along the chief axis. A clear hyaline apparently structureless layer over the entire surface

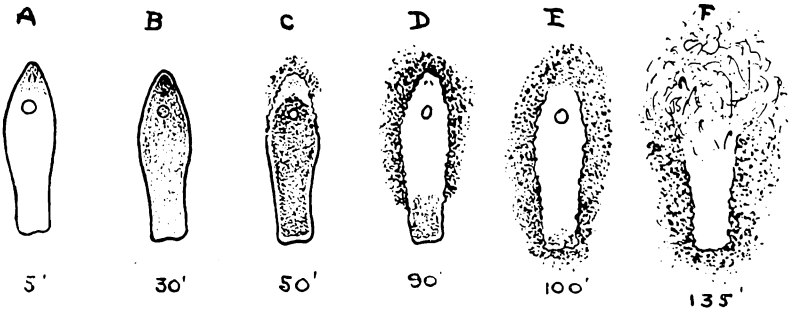

Fig. 5. The anterior-posterior gradient of staining *A*, *B*, *C*, of decolorization *C*, *D*, *E*, and of disintegration *E*, *F*, of *Phaenacora agassizi* in 1 per cent methylene blue.

remains relatively unstained. Almost at once the underlying tissues begin to stain around the sensitive point and edges of the reddish pigmented anterior end. It should be noted that this part lies well in front of the pharynx opening, contains no portion of the alimentary canal, and that no stain has yet become visible in the pharynx itself. The dye may be seen penetrating farther and farther caudad until in about 30 minutes its presence is indicated at the posterior truncate end. At this time the color differential is well marked; the pointed anterior end exhibits the first and the most abundant large blue granules which are progressively fewer posteriorly. Other concentrations give the same order of staining. The intensity of coloration continues to increase but the difference antero-posteriorly is never lessened until death changes are evident (fig. 5).

2. The gradient of extrusion of the stain. In about 40 to 50 minutes after exposure to 0.01 per cent m.b. the uncolored superficial layer in front of the pharynx assumes a somewhat bubbly outline as the cuticle is raised in small blebs. The edema or swelling in this zone doubtless implies changes in permeability and more or less local injury preluding death of the part, for closely following upon these alterations of state of membranes and tissues there ensues a conspicuous expulsion or escape of bluish granules and spherical clumps of cell material leaving the region without stain or pigment but surrounded by a blue halo or corona. The zone of extrusion slowly progresses backward and reaches the posterior end 45 minutes or an hour later.

3. The disintegration gradient. Immediately following the loss of colored particles a dissolution of structure sets in, manifested by further aggregation, clumping, swelling and lack of cohesion, fading away of the limiting epidermis and ultimate disorganization into semi-fluid transparent droplets or rounded granular masses. As superficial structures disappear and dissolve internal parts swell and push out. By the time colored particles are first thrown out from the intact posterior end, the anterior end is already disintegrating. At $2\frac{1}{4}$ hours disintegration has completely obliterated anterior structure and has been carried well toward the caudal end, the sound tissues being demarcated always by a sharp and well-defined boundary. Apparently in all cases the decoloration as well as the breakdown of tissue, once begun, is more rapid and simultaneous posteriorly, as if the gradient of susceptibility were more level and uniform there, though steeper anteriorly. That disintegration follows close upon death is indicated by the continuance of the ciliary stroke to the moment of disintegration when the beat becomes feebler and crawling and finally comes to a full stop.

Dalyellia (*Vortex*) *viridis*, also stains expels stained particles, and disintegrates in a definite anterior-posterior order.

Bothrioplana alacris? A small white rhabdocoel, evidently with triclad affinities, was collected from a temporary spring pond, but the sudden failure of the material left its identity uncertain. In 0.002 per cent m.b. staining begins definitely anteriorly, attacking the tip and especially the ciliated pits on the margin of the head. In 20 minutes the gradient is marked; at 30 minutes posterior parts are not yet stained, except sometimes at the extreme caudal end. In 40 minutes disintegration starts in ciliated pits and margins of the head and extends backward at such a pace that the anterior half of the

animal is removed in 55 minutes and the whole body scattered in 1½ hours.

Stenostomum leucops. As collected in April and May in indoor cultures this form consisted largely of chains of from 2 to 5 zoöids, but there were individuals in nearly all stages of fission and regeneration. Since a relatively high degree of differentiation (ganglia, ciliated pits, pharynx, etc.) is attained before zoöids separate from the chain, *Stenostomum* should be contrasted with planarians in which the posterior part is removed in a much less developed state.

A posterior zoöid of a 2-zoöid animal recently divided shows, if any, only a weakly developed secondary zoöid so that the gradient is simple and straight. The stain becomes visible first in the ciliated pits, then extends superficially to other more posterior levels. It finally seems to strike deeper and reach the ganglia underlying the pits, the other nerve structures, and sense organs around the mouth. Disintegration follows in 1 to 2 hours in 0.02 per cent m.b., usually before much stain is detectable posteriorly. Around the pits and over the main nerve masses the tissue swells even to rupturing, the protoplasmic masses taking deep stain when thus exposed. Hence in this case the disintegrating part is bluish. The ventral (oral) surface may disintegrate somewhat more rapidly than the dorsal surface.

An anterior zoöid recently detached from its posterior one stains and disintegrates at both anterior and posterior ends; the posterior end being both stimulated and exposed at the point of separation. Sometimes the fission end is lost well before the anterior end. In practically every instance disintegration of the anterior end follows the rule of midventral precedence over lateral and dorsal parts. In very acid media only the pharynx wall stains—in a sort of a network.

In an intact 2-zoöid animal the anterior end stains and disintegrates first; the new-forming anterior end of the second zoöid follows next in order, more rapidly if well formed, only after a time if not manifestly differentiated. A region where a fission septum is forming or where fission is taking place stains not only behind but also, and fully as much, in front of the septal plane. This region is doubtless subject to marked stimulation attendant upon the stresses and strains of the more or less violent separation which the dyes tend to induce. Individuals with more than 2 zoöids also tend to break up into discrete zoöids, but when the animal remains compound the zoöids stain as independently as in the 2-zoöid specimens.

Paramecium caudatum. Among protozoa *Paramecium* and *Dileptus* were chosen on account of their commonness in infusions, their elongate and axiate form, their comparatively exposed and uniformly ciliated surface, and the position of the oral aperture far back from the anterior end.

Most coarser stains, both basic and acid, are taken in and segregated in or near the food vacuoles of *Paramecium*, but m.b. and many other dyes of high visibility and marked color contrast also enter and stain somewhat diffusely even the less granular parts of the cell. With these agents, at 0.001 per cent or more, it is seen that individuals from actively dividing cultures show a distinct deep staining first in the extreme anterior end (fig. 6), soon concentrating below the ectoplasm in the outer endoplasm, in which the color spreads gradually backward; meanwhile the food vacuoles store much dye and collect posteriorly. In any effective concentration of the dyes the animals commonly

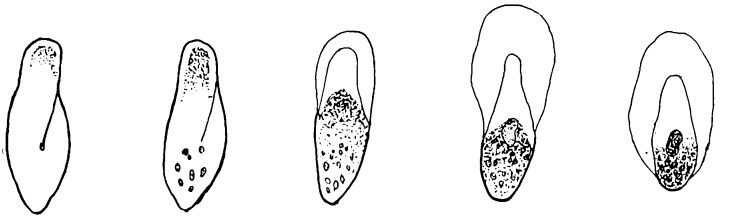

Fig. 6. Order of staining and disintegration of Paremecium in methylene blue.

reverse the direction of their swimming, or alternate in vigorous forward and backward movements, until they become sluggish and finally come to rest when they are heavily stained (except for the nucleus).

Shortly there occur changes in the gross appearance of the cell—the surface contour becomes more spherical, and the cuticle with its patterned markings, the cilia, etc., is lifted off the underlying parts and often ruptured, as if by inner swelling and the accumulation of a vacuole-like blister of fluid in some portion of the anterior end (not usually over the contractile vacuole). The fluid appears to force more and more of the more solid central contents posteriorly until the anterior portion of the cell has been practically emptied except of liquid while the caudal end is dense and crowded. The ciliary strokes cease soon after the outer layer is raised up, and always in the antero-posterior order, with many minutes intervening between their cessation in front and behind. It should be noted that only late in this process does the nucleus become deeply stained—a certain sign of lethal exposure.

This description applies quite generally for other basic dyes and for other typical ciliates, as *Stylonychia*, etc. Budgett (8) described antero-posterior dissolution of *Stylonychia* as a result of lack of oxygen, addition of KNC, pilocarpine, etc., and Child reported a somewhat modified gradient in several ciliates with KNC.

Dileptus gigas. This very large and elongate ciliate also reverses its direction of progression in the irritating dye solutions, until depression and paralysis ensue. In low concentration some basic dye is ingested and stored in the vacuoles, but in greater concentration little is thus taken in, and the animal gradually shortens and rounds out as it

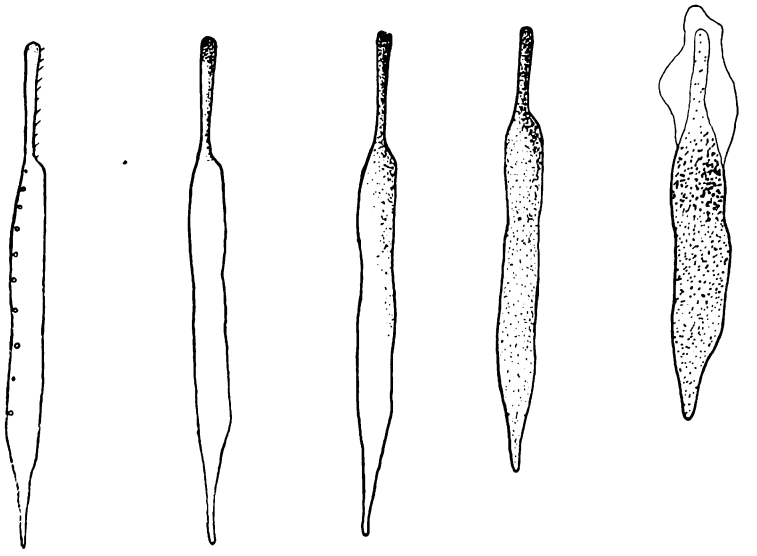

Fig. 7. The gradient of intravital staining and of disintegration of *Dileptus gigas* in methylene blue and many other basic dyes.

becomes quiescent. The stain enters first at or near the tip of the proboscis, especially along the row of large ventral cilia which extend back toward the mouth (fig. 7). Thence it continues to become visible further back in the middle regions and finally at the caudal tip itself. Disintegration occurs either slowly, or sometimes suddenly, with a loss of substance of the proboscis tip and base, of the oral region, and so on; or in some cases quite differently by a series of ruptures along the dorsal side opposite the many contractile vacuoles.

Hydra oligactis. Hydra is also instructive, in providing a case of a radially symmetrical animal where secondary budding may be followed in all stages (fig. 8).

A young hydra without buds, placed in a Syracuse dish, allowed to come to rest and attach for a time, and then covered carefully with m.b. (e.g., about 0.002 per cent), shows blue granules first in the ectoderm of the tips of the tentacles, on the mound of the hypostome, and on the body below the bases of the tentacles. The tentacles load up rapidly with stain, especially at their ends which soon surpass all other regions in their intense blue coloration. Dye meanwhile is detectable more and more basally upon the column and in a half-hour or more may be found everywhere in the ectoderm, excepting sometimes in portions of the base of the stalk. Nematocysts are commonly discharged instantaneously by strongly irritant dyes, and may be seen heavily colored throughout, either attached *in situ* or thrown out into the medium. They are practically always extruded first from the

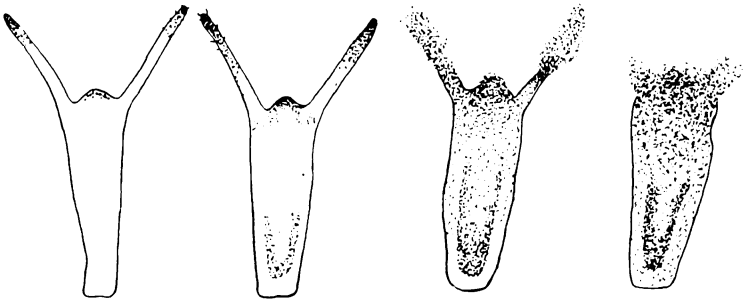

Fig. 8. Order of intravital staining and early disintegration of *Hydra oligactis* in many basic dyes.

distal parts of the tentacles, and then more synchronously elsewhere. (Incidentally, this action of irritant basic dyes provides an excellent method for demonstration and study of the kinds, numbers and distribution of the stinging cells, especially of the smaller kinds shown only indifferently well by acetic acid and methyl green.) When disintegration occurs it also begins distally in the tentacles, which are slowly removed bit by bit down to their bases, after which the hypostome and adjoining oral parts are similarly broken down. Basally there is less order and regularity in the disorganization. While the stain was entering the ectoderm it was also accumulating in the gastrovascular cavity in an irregular fashion, chiefly at the bottom of the cavity, and somewhat orally reaching out into the bases of the tentacles. It is this fact, that the dye is taken in by the large mouth and made available to the endoderm, which helps to complicate so much

the later stages of disintegration in larger hydras. But even this cannot conceal the essential fact that in hydra a gradient exists from tip to base in the tentacles, oral to aboral in the column as has been shown by Drzewina and Bohn (9) with lack of oxygen, heat and chemicals, and by Child and Hyman (10) with KNC and dyes.

If a well-formed bud is present, it takes the stain and disintegrates in about the same order as the young specimen. With regard to the time of first staining of the bud and the parent there is fair constancy of behavior; usually the parent tentacles stain earliest, accompanied or followed soon by bud tentacles, and then by the body of parent and bud. The rounded or cylindrical elevation where a new bud is forming, even though it be but a proliferating rudiment, exhibits a considerable capacity for early local staining. A bud eminence thus stains prior to the adjacent parent body. Disintegration of tentacles of a large bud and of the parent occur at about the same time; a small bud without tentacles is disorganized after the parent tentacles but before the parent body of the adjoining level is attacked.

Neutral red gave evidence of a similar gradient: from tip to base in a tentacle, and from hypostome downward on the column. To congo red and phenol red, which can hardly be said to stain, and even to hydrogen peroxide, hydra displays a like differential susceptibility for it succumbs gradientwise in these agents.

Hydra differs from most animals used in that it will take up and concentrate a basic stain from solutions so dilute as to appear clear.

Among the Annelids a number of common fresh water species were used. For purposes of comparison and confirmation data on these forms has fortunately often been available from the work of Hyman (11), who gives an interpretive analysis of the process of regeneration and demonstrates and describes the gradients of susceptibility to KNC in many oligochetes. It will be obvious that, except in minor respects, dyes show the same gradients.

Aelosoma hemprichii. This small form was collected readily from mixed protozoan cultures. The conspicuous structures are: reddish oil globules imbedded in the integument, and, apically, the flat rounded sensory prostomium, a ciliated pharynx, and cerebral ganglia just anterior to the pharynx.

Both staining and the ensuing disintegration proceed down a gradient of a primary sort (fig. 9). In an intact animal without fission planes the stain (e.g., 0.005-0.01 per cent m.b. for 1 to 4 hours) shows first in the ciliated pits and thickened sensory epithelium of the rounded ventral

surface of the prostomium and in the oral epithelium, but is soon visible also in more dorsal areas above the mouth between the pits and over and in the two ganglia lying superficially in full contact with the epidermis. From the deeply colored anterior end the staining area (and disintegration later) is carried backward at first slowly behind the mouth and then in more rapid sequence through the more posterior levels. In this progress stain does not enter segment by segment but in continuous gradation. The oil globules appear to stain about equally rapidly at all levels. The ciliated pharynx and the oral parts of the intestine may draw in and accumulate quantities of the dye, and produce a local area

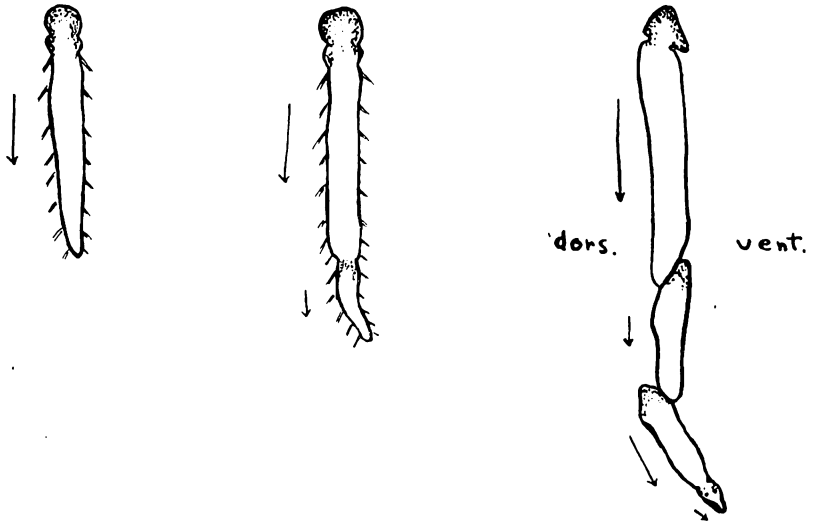

Fig. 9. *Aelosoma hemprichii*, showing order of staining of different specimens, composed of one, two or more zoöids.

of deep staining disturbing the simple gradient, but the first parts actually to stain lie wholly in front of the alimentary canal and out of communication with it. The posterior end stains early if a posterior zoöid has recently been removed.

If an animal possesses a marked fission plane, the stain enters in a definite ring of epidermis at or near each anterior end and concentrates, especially on the dorsal surface, behind the plane. The further course and times of staining are similar in both zoöids, which are ordinarily separated by the disintegration of the anterior end of the second.

Dero limosa. The important structures and their order of staining are shown in figure 10. In 0.01 per cent m.b. a sound specimen without

zoöids stains quickly in cutaneous portions, particularly anteriorly in the prostomium and ventral sensory buccal areas. An especially active ciliated gill region at the anal end composed of gills in a respiratory pit colors about as soon. Staining progresses slowly back from the head and more rapidly from the anal end forward. In this latter course it moves by segments or small blocks of segments, which stain first near the septa where, intersegmentally, a sphincter-like contraction takes place and forms a chain of blue bead-like rings, such a chain lengthening by additions from in front. In each segment the deepest mass of dye accumulates in ventral patches apparently corresponding in position to the segmental ganglia. The advance cephalad is soon met by

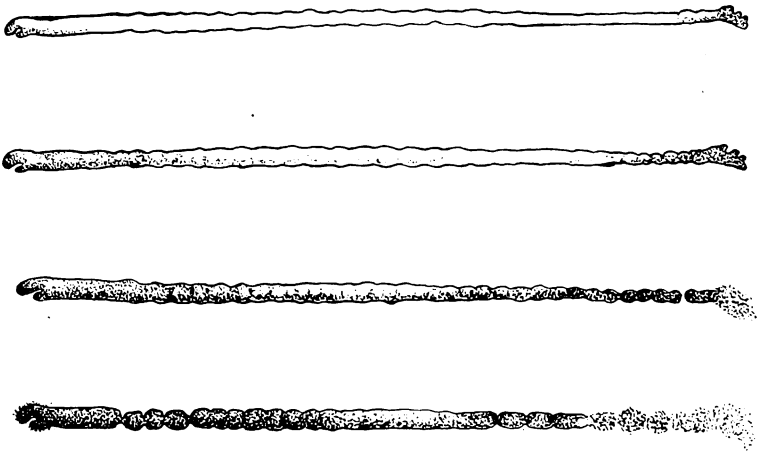

Fig. 10. Progress of staining and disintegration in *Dero limosa*, resembling generally that for most higher Oligochetes.

anterior staining moving caudad. In this and the higher annelids the cuticle seems to interfere with the penetration and fixation of the dyes, for in all staining is uneven at any given level and may wash out for some time after it has passed through the outer covering.

When two zoöids are present, the anterior stains the more darkly at first and in the antero-posterior order. A posterior zoöid stains less deeply, at first postero-anteriorly and later also from its anterior end back. As differentiation of cephalic structures proceeds in this zoöid these stain more and more quickly, finally equalling or even anticipating the anterior end of the first zoöid.

The first losses by disintegration are from the gill region and several of the posterior segments. Then follow the prostomium and the next

succeeding anterior segments. By the time the head goes the last 10 or 12 segments have been cut off one by one or in small groups. Intermediate portions may recover partially after losses from either end.

Lumbriculus inversitans. In 0.001 per cent m.b. epidermal structures stain chiefly at the anterior end and the caudal tip. The head stains quite uniformly along its length; it lacks septal divisions. Dye is soon visible more or less irregularly in intermediate regions, and edematous swelling occurs at corresponding points.

A part becomes pale blue on disintegration. The caudal third or more of large worms, beginning posteriorly, has formed a bead-like series of blue segments, as in *Dero*, by the time the head shows first signs of actual breakdown. The wave of further disintegration spreads backward from the head and forward from the anal end. Segment by segment the stain floods into the tissues and then is partly lost again in the subsequent dissolution process. Smaller and larger worms begin disintegration about simultaneously, but smaller ones may complete it sooner.

Tubifex tubifex and *Limnodrilus claparedianus.* Cutaneous parts, bristles, as well as the whole superficial nerve plexus take on stain early. In small, 1 inch specimens the anterior end colors first. In larger ones before the head segments stain perceptibly posterior ones have long since colored deeply and often broken off. In *Tubifex* where the cuticle is thinner posterior segments drop off one at a time; in *Limnodrilus* they detach in sections. Meanwhile the whole anterior tip loads heavily with stain. Prior to the sloughing off of segments the purplish stain seems to concentrate heavily along the lateral portions of the segments and then spread elsewhere; otherwise anterior rings stain most prominently at the bristle level and posterior ones in blue bands intersegmentally. Finally the dye concentrates ventrally in each segment. The last parts to stain are the dorsal portions of those segments somewhat behind the head. Rhythmic pulsations of blood vessels and alimentary canal continue regularly until slightly before the deep staining of the approaching death-point is attained.

In disintegration the parts swell greatly and constrict between segments. Usually the posterior half is lost by detachment of rings or groups before the head dies; even in 0.001 per cent m.b. 20 or more anal segments were dropped in $3\frac{1}{2}$ hours. Head and succeeding parts go next, the last surviving segments being a block about one-fourth of the distance back.

The chick embryo. The incubated egg was opened and immersed in a solution of 1 part m.b. powder in 5-10,000 parts of isotonic saline, previously warmed to incubation temperature and held at about that point for the course of the experiment.

The blastodisc, germ wall, and actively growing vascular area stain before the yolk or vegetal pole. The regions most sensitive to coloration are the formative regions: the edges of the uprising medullary folds in the anterior parts, and the sides of the neural groove posterior to the head fold, fading out more posteriorly until the level of forming somites is reached and back toward the anterior end of the primitive streak, where deeper stain is again met with.

On 3-somite chicks the medullary folds and head ectoderm are the most conspicuously stained portions anteriorly, and the end of the folds posteriorly, where some active growth and proliferation is taking place.

Five to seven-somite chicks stain deepest on the head, the intensity thence grading down in the region of formed somites and then gradually rising again in the extending medullary plate and folds. It certainly seems to be clearly possible to distinguish here a descending primary gradient (of differentiated structure) and a posterior ascending (growth) gradient, resembling generally that in annelids. At least the ectoderm of a 10 to 12-somite chick becomes a deep opaque blue-black in the head, which fades out to the end of the region of the closed medullary tube, whence it again deepens toward the posterior ends of the medullary folds. The trough of the medullary groove is comparatively lightly stained. The bulk of the stain accumulates at first only in the ectodermal layer, for this may be peeled off leaving the interior practically uncolored. The mesodermal somite regions, however, evidently show a somewhat similar gradation of superficial staining later, lowering from the head through the formed somites and rising sharply toward the posterior end of the region of developing somites and anterior to the unsegmented plate.

DISCUSSION

In at least three ways the intravital dyes might be expected to contribute to a solution of the problems of differential susceptibility and to a closer study of the real nature of a "dominant region" or active state. (1) The movements of their conspicuously colored particles may be followed more readily than those of most agents, their points and rates of penetration and sites and manner of storage noted, and the facts so

obtained correlated with the results obtained by the susceptibility method with the dyes and with other agents and conditions. (2) Alterations in penetration and storage may be studied: *a*, during different functional states; and *b*, with the dyes in media containing dissolved salts, acids and bases. (3) Use may be made tentatively of the physicochemical properties of the dyes and of protoplasm, as far as known, for analysis of the process of staining and of the manner of production of dye effects upon the organism. This applies to dyes, toxic and relatively non-toxic, vital staining and non-staining, as well to those undergoing some definite change (oxidation, reduction, or a change in color indicating reaction, or a change of state) as to those which apparently resist such changes. Obviously this work makes use of by no means all the possibilities of the method.

1. The susceptibility gradient with dyes. The general significance of the differential susceptibility existing along the structural axis of axiate organisms has been often and fully discussed (2),(11). In the light of the data derived largely from these previous studies with KNC, lack of oxygen, and narcotics, and from the quantitative determination of gas intake and output, there seems to be full warrant for the belief that, under conditions where death occurs at all rapidly, the relative susceptibility indicates more or less delicately a gradient in some slight differences in metabolic activity, associated in all probability with oxidations.

As described in the details of this paper, the numerous basic and rarer acid dyes which are toxic enough in nearly neutral solution to be distinctly lethal, produce disintegration beginning in cephalic parts and other sites of known markedly high metabolic rate—this disintegration agreeing, so far as the facts have been determined, in fundamentals with that for cyanides, etc., in the same forms. The chief divergence in results occurs in the lack of delicacy of the dyes in distinguishing the small differences detected by KNC. Even such dyes as m.b., which undergo oxidation and reduction changes, failed in the same way and were disappointing, inasmuch as at the beginning of the work it was thought likely *a priori* that they might give positive results. Helpful facts should certainly be obtained from the use of methylene blue and its leucobase in conditions where oxygen can be supplied or withdrawn at will.

The nature of the difference in susceptibility to dyes as compared with that to KNC, etc. is interesting and perhaps significant, particularly if compared also with susceptibility to acids and bases (4). It may be

fairly stated that in this matter and in general physiological effects basic dyes behave much like acids (HCl or acetic), and acid dyes much like alkalies (NaOH). Thus both basic dyes and acids produce in flat-worms a most rapid disintegration of the anterior end, often of the ventral surface as compared with the dorsal, sometimes of the midline as compared with more lateral parts; the anterior ends of the second and succeeding zoöids are not singled out for early disintegration; no matter how low the concentration and how slow the killing, disintegration, when it does occur progresses posteriorly; and finally, in any quickly lethal concentration the young and old individuals are nearly equally tolerant, the young being but slightly, if at all, less resistant than the old. Acid dyes, conversely, resemble alkalies in their effects, though seldom sufficiently toxic to be readily lethal except in solutions made considerably acid. Thus alizarin blue S causes most rapid disintegration of the anterior end, dorsal surface and lateral margins, and often of the anterior ends of the second and third zoöids; and young individuals are plainly much less tolerant than older ones; and in certain cases indirect susceptibility was observed.

These similarities of action had not been anticipated, in fact were discovered entirely empirically, but might logically have been expected from the likeness of the dissociation of these agents. The neutral salts of basic dyes hydrolyze freely in neutral or alkaline solution, and dissociate into a large colored basic ion, the cation, which moves toward a cathode, and the anion radical, e.g.,Cl', of a strong acid. Evidently *the large color ion, like the mobile H ion, predominates powerfully in effect over the anion.* On the other hand, acid dyes dissociate, if at all, into a large colored anion resembling hydroxyl ion in effects, and a less potent metallic ion like Na^+.

The influence of impurities, such as heavy metals, in these dyes deserves treatment, of course, but these did not seem to interfere with the action above described, which I interpreted as due to the dye itself, since all samples of numerous dyes from varied sources gave like effects. If acids are added to acid dyes or alkalies to basic dyes to increase their action the results are naturally neutralized and confusing.

2. Decolorization gradient. That a gradient of decolorization by extrusion or escape of dye and dye particles should be found in some forms, as many flatworms, is not really a new observation. Such loss of stain, like the loss of natural pigments many times reported occurs commonly when death is imminent, and may be taken to indicate that resistance to the passage of diffusing substance has disappeared, and

conditions of normal semi-permeability destroyed, or perhaps, in this case, rather that retaining surface layers have been broken and loosened by beginning disintegration, and allow escape of discrete particles, especially if these are under pressure from adjacent swollen tissue or from a cover slip. Recovery is, however, often still possible after a certain amount of this loss has taken place.

3. The staining gradient. Although a sort of coloration gradient had been reported by Child with potassium permanganate (12) and with indophenol (3), the field of true axial protoplasmic vital staining with dyes has been entered deliberately only in this and associated studies on algae and hydroids (13); hence the facts merit brief discussion here.

Any doubt of the general observation that with easily visible basic dyes, such as methylene blue, toluidin blue and many others, there is produced, as reported for the forms used, a color gradient corresponding approximately to the major susceptibility and metabolic gradients (as determined by HCl rather than by KNC), may be removed by a few simple but convincing experiments such as above outlined, with organisms consisting either of one zoöid or of two or more morphologically distinct zoöids. Generally speaking, the order of staining of parts corresponds to the order of their susceptibility to acids and to the basic dyes themselves. Slight differences of susceptibility as shown by cyanides are not usually indicated either by susceptibility or by staining with these dyes, but it is safe to state that, knowing the order of staining with basic dyes one can quite reasonably predict the general course of disintegration with most lethal agents, or knowing the relative susceptibility of parts, their relative staining times may be foretold.

Concerning the correct interpretation of these findings there may well be some diversity of opinion. Where so little is really known one may not safely become assertive or dogmatic. But I shall point out a few indications of the rôle of some physico-chemical factors underlying, conditioning, or associated with metabolic gradients, to this end invoking the aid of current doctrines. To attempt to apply the classic as well as more recent theories of vital staining, both "physical" and "chemical," may help to recognize, eliminate or evaluate the factors here concerned.

First of all, one may be assured that there is no simple gross anatomical explanation to account for the axial ingress of the stain. The mouth is seldom situated at the cephalic end of the staining gradient, and is often well back, e.g., midway in flatworms, and at the base of

the tentacles in hydra. The rôle of the alimentary canal as a path of staining is easily recognized, as in the cases of *Hydra* or *Aelosoma*. The first parts to dye are usually particles or globules in or subjacent to the epithelial surface and stain passes in through the surface not by any large aperture. Nor is there staining of some special tissue alone, though ciliated and sensory surfaces and nervous elements are oftenest first conspicuous. The gradient is easy and continuous, but need not be always antero-posterior, as differentiated structure would commonly be; in higher annelids the dye enters earliest at the ends, and later in the middle regions. Much the same limitations apply to a "density" factor. If metabolic activity leaves an accumulating residue of organized reserve or inactive stabilizing substance, then as differentiation proceeds this material might be laid down gradientwise and be stained accordingly; but a newly regenerated head, highly transparent, nongranular, and relatively undifferentiated accumulates stain earlier than adjacent posterior levels, and the staining particles appear identical throughout the axis.

In view of Ehrlich's demonstration (7) of the relation existing between staining capacity with methylene blue and the rate of oxidation in different tissues, the assumption was made *a priori*, that stain might enter equally all along the chief axis but become invisible in certain parts through transformation into colorless base by marked local reducing action. That such is not the case here is shown by tests with strong oxidizing agents, as H_2O_2, which fail to reveal, i.e. make blue, any such invisible dye base. On the contrary, there is every reason to believe that a greater power of reduction in deoxygenated water must be possessed by those most active parts, first staining in aerated water: and it seems likely that in deficiency of oxygen supply reducing power is a criterion of vital activity, while in abundance of oxygen staining with methylene blue is such a criterion. Certainly in well oxygenated media any local reducing action may be ignored entirely, especially since in most of the vital stains the colorless form does not exist.

The gradient may, of course, be "explained" and dismissed as due simply to differences along the axis in *permeability* of the membrane to dye particles. If by this term one implies some kind of ultrafilter in the sense of Ruhland (14), then it may I think be rejected, for the size of particles of basic dyes is increased in the same alkaline solutions that facilitate their penetration and the same is true for acid dyes in acid solutions (6). In any case the mechanism of alteration of permeability itself requires analysis.

For the popular Overton theory (15), maintaining that the entrance dyes, basic or otherwise, into nervous and other tissues is determined by their relatively high solubility in lipoids that collect at surfaces and phase boundaries, there is no support here. The theory obviously cannot hold both for the lipoid-soluble dyes (janus green, dahlia, neutral red, methylene blue, methyl violet) and for the similarly acting lipoid-insoluble dyes (toluidin blue, thionin, methyl green), all of which enter and exhibit strikingly like effects in the cases tested. The intravital stains are mostly basic, and many of them have been designated as "specific" nerve stains, but even for the nerve it is not the myelin sheath which is colored, but the neurofibrillae and Nissl bodies (16). Solubility in lipoids would appear to have no prominent influence in controlling distribution or effects of dyes as here described.

Ehrlich and his students, Fischel (17) and Goldman, contended that vital dyes react chemically with definite specific dye-receptors of large protoplasmic molecules, which receptors might conceivably concentrate in graded amounts along the axis and could thus determine and measure the relative affinities of various parts of the animal for the stain. It is hard to refute this view but many facts stand against it as stated in its original form: first, nearly all or all levels parts and tissues of the organism stain *finally* to approximately the same depth; it is merely a difference of time required to bring in the stain and make it visible, not of stainability or of amount of stain taken up ultimately. Second, the marked non-specificity of staining with the various basic dyes points to some more fundamental common property conditioning the reception of the stain. Chemically unlike dyes (thiazins, azo-dyes, etc.) (5), (18) often behave alike, and those of closest chemical relationship are as frequently opposing in effect. Further, practically any lethal agent or condition will produce a disintegration gradient of an essentially similar nature, differing only in minor respects-cyanides, narcotics, lack of oxygen, excess CO_2 or other waste products, salts of heavy metals, dyes and indicators, ions of electrolytes, high and low temperatures, and doubtless a great variety of other unfavorable conditions and of agents of no known or obvious chemical similarity or kinship. As to the dyes, the most prominent cleavage among them is not one of specific chemical constitution but that between acid and basic, a matter of reaction and manner of dissociation. Admitting the probability of some specificity in details of working of individual dyes does not warrant speaking of staining as a specific chemical process. There must be some widespread and less specific chemical or physical character responsible for the course of basic vital staining.

In the writer's opinion the staining gradient may well be due to the greater entrance and fixaton of dyes in certain regions corresponding to a graded difference in adsorption or combining capacity, itself based on metabolic activity. This conception is not inconsistent with the belief that continued entrace of the dye depends in large part on its power of combining with or being precipitated of flocculated by certain constituents of the cells, and that its accumulation is possible because more or less insoluble compounds are formed within (19).

Valid evidence as to the mode of fixation of the dye may be obtained from the study of the conditions necessary for staining of textile fibers, proteins, etc., *in vitro* and *post mortem*. Basic dyes, yielding electropositive colored ions, form insoluble colored salts with many "acid" protoplasmic substances containing organic acids combined with strong bases, e.g., mucin, hyaline cartilage, nuclein, amyloid, casein, Nissl bodies, yolk material, soaps, etc., the dyes combining like metals to form a basic-dye-albuminate, etc.; but basic dyes will not combine with the more basic or neutral albumins, globulins, albumoses, histones, etc., except in alkaline solution. These latter substances, however, especially in acid media, combine readily, often with a precipitation, with free inorganic or organic acids and with acid dyes of all kinds, giving an albumin-acid-dye compound (20), (21), (22). Neutral gelatin combines with neither acid nor basic dyes; if made electropositive it stains with and retains acid dyes (acid fuchsin), and if made electro-negative it takes up and retains basic dyes (neutral red) (23). In general, previous adsorption of acid or neutral salt ions tends to discharge and aggregate electro-negative colloids, and accordingly both *in vivo* and *in vitro* decreases basic staining and increases acid staining, while conversely, alkaline solutions favor basic staining and diminish acid staining (5), (24), (25), (27).

These suggestions by way of an electro-chemical view of staining are supported by the facts of vital staining. Methylene blue and neutral red form insoluble dye-tannate compounds with tannic acid of the sap vacuoles of *Spirogyra* (28); neutral red forms a soluble red compound with some organic acid in *Elodea;* while in animal cells, as in Paramecium and in Echinoderm eggs union is made probably with some lecithoprotein (29). From recorded and new data Von Moellendorf (27) concludes that basic dyes react with natural acid colloid (anion) constituents of cell protoplasm in the same manner as they react with acid dye ions either *in vitro* or stored previously as granules *in vivo;* but basic stained protoplasm cannot thus combine later with acid dyes!

Matthews believes that basic dyes stain because they form some insoluble compound (salts, esters, etc.) with the "proteinate," "lecithinate," amino-acid, cholesterol, fatty acid, and other similar ions of protoplasm. The compounds may be regarded as weak surface chemical combinations quite insoluble and inert chemically, usually but not always highly stable, sometimes slightly dissociating and partly reversible.

That animal protoplasm is usually electro-negative is attested by both electrical and physiological observations; under ordinary conditions and in ordinary slightly alkaline media the proteins and lipo-proteins of which it is composed, having low iso-electric points, dissociate with negative charge (5), (30). Electro-negative colloids behave electrically like anions of an acid, and anions combine with or adsorb positively charged metal or basic dye cations, but not acid dye anions, which wash out leaving no stain. Even a prolonged immersion in an acid medium does not commonly, but may sometimes, suffice to bring about acid dye staining. In the exceptional cases where colloids are positively charged, as in hemoglobin of erythrocytes, perhaps in many plant cells, and apparently in some leeches, it is the acid dyes that really stain. In extreme acidosis also tissues may stain vitally with acid dyes (31). From this view the likeness of the effects of basic dyes and acids, both with predominant cations, is to be expected.

If anions or acid radicals are important requisites of basic staining, then there would need be a graduated production of such anions along the axis to account for the staining gradient, as well as a greater abundance of them in certain tissues, as nervous tissue, and inactive parts generally. A constant, and in life unfailing, source of these anions may be sought in the katabolic processes, which yield acid products always on the whole preponderating over the ammonia produced. From split products an increased number of molecules results which, like amphoteric substances in the alkaline interior of the cell, dissociate as acids with a maximum number of anions and a certain number of H ions. This would not necessarily result in any actual acid reaction even locally; one can only speculate as to the disposal of the H ions, whether by neutralization, or by rapid escape outward, or by formation of a Helmholtz double layer; rapid liberation of H ions may produce the galvanometric electro-negativity of the active part (32).

It is interesting to note what has been taken as a curious old observation, that only the color ion of a basic dye is adsorbed, while the Cl ion is left in the outer solution (5). Similarly only the H ions of an HCl solution are absorbed, according to Gray (33), who says that the

charge of the Cl′ outside is satisfied by outgoing K ions, which are themselves replaced by H ions. It seems likely that basic dye ions likewise take the place of H ions, providing these are able to make their escape into the outer medium (as in neutral or alkaline media), but if the H ions are unable to escape (as in acid solutions without, or with acid reaction within the cell) they would block the entrance of the dye ions. For there is considerable evidence that if reaction of the cell actually becomes acid it no longer stains with basic dyes (6). In general it is sufficient if one of the results of rapid metabolism is a correlative net increase, momentarily at least, in negatively charged ions, which constitute the basic staining substance or condition. Naturally if the anions are formed by dissociation of amphoteric proteins as acids, their production should be reduced by acidity and facilitated by alkalinity, by abundant oxygen, rapid diffusion of CO_2, etc.

The rate of entrance of a dye may be controlled not alone by capacity to form compounds within, more or less depending on metabolic activity; conceivably a greater water content of active parts may aid in more rapid diffusion, or katabolic acids may lead to a more aggregate or more swollen condition, which may stain more rapidly. And there are doubtless other unanalyzed factors in the graded permeability along the axis; in the next section high permeability to dyes will be seen to be practically always associated with states of stimulation and activity.

I believe the points to be not witnout significance that all of the dyes of whatever chemical consitution and however varied the details of their staining pictures, were always in substantial agreement as regards the parts first stained as well as in the final staining gradient; that the dye first become visible at the same definite loci (e.g., the auricles of *Planaria*), whether staining exactly the same substance or not; that these loci are practically always the loci of greatest general susceptibility; that all of the really successful vital stains were basic, while the acid dyes were as generally signal failures; and finally that this staining is apparently dependent upon metabolism, since previously killed animals show no staining gradient.

4. The essential similarity of protoplasmic condition in the dominant region, a stimulated region or condition, and in a fertilized egg. Of more than ordinary interest are the variations in staining in different physiological states, particularly that of excitation. For instance, recently traumatized regions, stomach ulcers (34), and healing wounds are characterized by a state of enhanced metabolic activity, increased res-

piratory exchanges, by weakening of the surface layers, and accordingly are stained easily and strongly.

There is in the literature considerable direct evidence that capacity for taking and holding basic stains varies to some extent with metabolic and respiratory activity. For gland cells the facts are fairly complete and all in full agreement. Asher and Garmus (35) and Garmus (35) showed by direct continuous observation that gland cells of the nictitating membrane of the living frog take up much more vital dye of all various degrees of solubility in lipoids (m.b., neutral red, rhodamin, bismark brown, toluidin blue, thionin) when stimulated to great functional activity by pilocarpine than when left unstimulated, and more when left unstimulated than when function was depressed by atropine. After injection of pilocarpine the secretory granules, etc., were more quickly colored, and with time their coloration became strongly intensified. After local or general atropinazition coloration began very late and never became more than weak, although the cells were as richly granulated as resting or normally functioning cells. The difference could not have been due, therefore, to a greater secretion, but must have been brought about, according to the authors, to an alteration in the permeability of the membranes. Keleman has since shown (36) that pilocarpine increases respiratory exchanges and CO_2 production as much as 10 per cent and increases CO_2 of both arterial and venous blood, while atropine diminishes these below normal. Pilocarpine also raises the relative galvanometric electronegativity of gland cells stimulated by it.

For nerve tissue a similar condition apparently obtains. Bethe (16) discovered that during and shortly after the passage of a polarizing current through a living nerve the neurofibrillae lose absolutely all power of primary staining with methylene blue or toluidine blue at the anode but increase their normal capacity for the same dyes at the cathode. After recovery is complete the staining polarity disappears. These physiological changes exactly parallel the electrotonus changes: decreased irritability and conduction at the anode (anelectrotonus) and increased irritability at the cathode (catelectrotonus); that is, primary stainability varies directly with irritability and conductivity. Bethe came to believe after some experimentation with alkalies, distilled water, narcotics, etc., that at the athode there is an increased affinity of the neurofibril protoplasm for a "fibril acid substance," which moves toward or otherwise becomes more abundant and firmly attached at the cathode pole and there makes the fibrils more stainable. Frequent stimulation, strychninization, and a relative predominance of

katabolic processes over anabolic gives a cathodal appearance to staining fibrils; prolonged rest or excessive stimulation gives an anodal appearance. In death and deep narcosis no polarity is produced. According to the view here suggested the cathode may be conceived as stimulating the production of metabolic acids and attracting and withdrawing from the fiber positive ions, in whose places may be substituted the basic dye ions. Neurologists have repeatedly found that an increased amount of basic staining occurs, e.g., in Purkinje cells of the cerebellum and other motor cells, after first stimulations, but that with excessive stimulation ending in fatigue and exhaustion the staining substance or condition vanishes and less and less staining occurs. Pollock and Cluney (37), commenting upon the results of intravital staining of brain cells of mammals with various dyes introduced into the blood stream or under the meninges, say that "any procedure which increases metabolic activity of cells insures a greater degree of intravitam staining" or ingestion of trypan blue.

Matzumoto (38) reports that very dilute (0.00001 per cent) neutral red or nile blue sulphate after 10 hours to 4 days stains characteristic granules of corneal epithelium of the frog. He notes a general parallel between activity of cells and presence of stain, the deeper basal swollen and active cells (which are more acid, according to Unna (39)) staining in many granules of all sizes, while there is progressively less staining in more superficial cells, and none at all in the flat polygonal cells of the surface.

There are many points of likeness, also, between the processes of fertilization (initiation of cell division) and of stimulation. Arbacia eggs shortly after either natural or artificial fertilization show a two to three fold output of CO_2 and of heat, a vastly increased O_2 consumption, a diminished electrical resistance (as in working striated muscle (40), (30)), an increased permeability to salts, water, natural pigments, and an enhanced stainability with methylene blue and neutral red (41). The most deeply staining individual eggs divided first.

In the light of new observations and relevant literature basic vital staining of different cells and tissues and of different parts of an axiate organism may be understood tentatively as indicating differences in staining condition more or less paralleling metabolic activity, which produces substances with anion radicals capable of combining with positively charged ions. It is yet impossible to clearly dissociate this combining capacity from accompanying and secondary (?) differences in water content, ion content, and especially in "permeability." It has

become increasingly obvious as this work progressed that a sharp distinction could hardly be drawn between chemical reactivity, combining capacity, and permeability; and indeed a semi-permeable membrane is not necessarily a definite, physically detachable, primarily static and unchangeable structure, but should be and commonly is (5), (30), (42) regarded as an integral part of the protoplasm, undergoing all of the changes characteristic of living substance, and hence showing with it similar modifications by agents in the medium as well as its alterations during functional activity. In fact concentrated and exposed surface protoplasm almost certainly participates in these changes even more quickly and completely than less available, remoter, interior parts, and the preliminary effect of an agent or physical condition (in compounds produced, altered or destroyed, or in aggregation and solution effects, etc.) may well facilitate or retard subsequent action and penetration of the agent, or effectiveness of the physical condition. Thus surface metabolism and condition may control general metabolism.

Semi-permeability is probably maintained by metabolic activity. It is interesting to see that organisms killed by heat or alcohol stain quite heavily in a fraction of the time required by a living individual, and in the dead animal no gradient appears, but intake of stain is quite uniform throughout, special sites of election being to all appearances absent.

As staining and susceptibility follow each other closely in their respective courses, it is readily conceivable and probably true that, though cells apparently uninjured and with unaltered membrane take up the stain, a state of excitation, such as we must suppose exists in a dominant part, amounts substantially to a mild injury; that there are all degrees of injury up to death itself; and that staining, like permeability, increases in general after injury and as death approaches.

A region or state of dominance in an axiate organism is thus characterized by possessing distinct differences in susceptibility, in regenerative capacity (2), in respiratory activity (3), (43), in electrical potential (32), probably in catalase content or activity (44). Evidence of differences in rate of vital staining or in permeability has been presented in this paper. In most of these properties the dominant region resembles any metabolically active or stimulated part. Whether it resembles an actively functioning part in certain further respects is yet to be determined. An examination for high water content may be made by analysis; heat production may be measured in favorable forms by some thermocouple device; and electrical conductivity may be quantitatively determined.

In view of the exact and extended parallels which may be drawn between the physiological state of the dominant region and that in any stimulated region it is helpful to conceive of the dominant region in general as a portion in a condition of more or less permanent tonus or continuous partial stimulation, more highly irritable and more quickly responsive than less active parts, and therefore as always activating or stimulating subordinate levels. It responds to stimulation after a briefer latent period, or period of restitution and recovery. Since its activity is but little arrested after responses it is relatively non-fatiguing (like nerve as compared with muscle), and because of these attributes it possesses to a greater degree than other parts automatism and spontaneity, the capacity of "initiating" impulses.

SUMMARY

These researches were undertaken to determine further the nature, characteristics and mode of action of metabolically active parts or tissues, especially those of the "dominant" (cephalic, apical, anterior, etc.) region, chiefly with the aid of a representative series of stains.

The methods employed were those of direct susceptibility and differential vital staining.

1. Both basic and acid dyes were used, but with few exceptions basic dyes alone were found to be sufficiently penetrating and toxic in nearly neutral solutions to be effective.

2. Neutral salts and H ions in the medium retard visible staining with basic stains; OH ions and higher temperature facilitate it, as do also local injury and local healing.

3. Data are given, especially for methylene blue, demonstrating more or less satisfactorily: a staining gradient, sometimes a decolorization gradient, and always a disintegration gradient in the forms used, including *Paramecium, Dileptus gigas, Hydra oligactis*, several flatworms and oligochetes; and a staining gradient for the chick embryo. The loci of highest general direct susceptibility to most agents and conditions are first to stain visibly with the dyes, first to decolorize (in case color is lost) as death approaches, and first to disintegrate at death.

4. The disintegration gradient with basic dyes resembles that obtained with acids; disintegration with lethal acid dyes resembles the type obtained with alkalies and KNC (p. 361).

5. Basic dye color ions are positively charged, and evidently are taken up by negatively charged colloids and other anions, which there is

reason to believe are most numerous in metabolically active regions, where acid substances are produced (pp. 378–380).

6. There is much collateral evidence to the effect that a difference in permeability accompanies states and sites of greater metabolic activity; but there appears to be little justification as yet for distinguishing sharply between combining capacity and penetration power, or to attempt to give priority to either.

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