DIRECT DEMONSTRATION OF THE EXISTENCE OF A METABOLIC GRADIENT IN ANNELIDS

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THREE FIGURES

INTRODUCTION

For a number of years evidence has been presented from this laboratory concerning the existence of metabolic gradients in organisms. This evidence has been obtained through the use of several methods. These may be summarized briefly as follows:¹

1. *Regeneration method.* In pieces cut from different levels of the axis of simple organisms the rate of regeneration and the kind of structure regenerated exhibit graded differences correlated with the level from which the pieces are taken. These differences in the regenerative phenomena at different levels cannot be due primarily to morphological factors, but are functional in nature, since they depend upon the size of the piece and are easily alterable by conditions whose action is chiefly quantitative.

2. *Direct susceptibility method.* Different levels of the axis of simple organisms exhibit a differential susceptibility to concentrations of toxic substances which will kill within a few hours. Since the death gradients of organisms in such solutions are the same for a large variety of toxic substances of widely different chemical constitution, these gradients cannot be due solely to the specific mode of action of these substances, but must depend rather upon a general quantitative gradation of some sort existing within the organism itself. The suscepti-

¹ For a more complete discussion of these methods together with references consult Child ('20).

bility method is also used to control morphogenesis during regeneration or development. For this purpose less concentrated solutions are employed which do not kill, but depress. This depression is differential; consequently, when regenerating pieces or eggs or embryos are exposed to the proper concentrations, morphological modifications result which are predictable and explicable on the basis of the susceptibility gradient. That differential susceptibility to toxic solutions is correlated with differences in metabolic rate is shown by the fact that conditions which are known to modify metabolic rate also modify susceptibility and in the same direction.

3. *Indirect susceptibility method.* The death gradient of organisms in dilute concentrations of toxic substances such that death occurs only after several days' exposure is exactly the opposite of the death gradient in quickly lethal solutions. This shows that the susceptibility results are not due to differential permeability, since in that case it would be impossible to account for the difference in the action of solutions of different concentration. The reversal of the gradients in dilute solutions is believed to be due to differential acclimation. Differential acclimation also accounts for certain modifications induced during regeneration and development exposure to the proper concentrations of toxic substances.

4. *Indophenol method.* When organisms are exposed to a mixture of dimethyl-*p*-phenylene-diamine and α -naphthol a blue precipitate of indophenol is produced. It is generally accepted that the formation of this precipitate depends upon the presence of oxidative processes. The depth of color produced along the axes of organisms through this reaction exhibits the same gradation as evidenced by the direct susceptibility and other methods. This method has been used only for embryonic stages and small forms.

5. *Potassium permanganate method.* When exposed to solutions of potassium permanganate organisms reduce it to manganese dioxide. The capacity to carry out this reduction exhibits the same gradation along the axis as appears by other methods.

6. *Electrical methods.* With the aid of a galvanometer it is found that permanent differences of potential exist along the axes of animals. These electrical gradients correspond to the susceptibility, staining, and reduction gradients, the regions of highest susceptibility, greatest staining, and reducing powers being electronegative (galvanometrically) to regions of lower susceptibility, staining, and reducing powers. The galvanotactic behavior of at least the simpler organisms is also in accord with the metabolic gradient conception.

The concordance of results obtained by these various methods indicates clearly enough the existence of a definite gradation along the axes of organisms. The nature of this gradation is naturally open to discussion. Our explanation that the gradation is primarily quantitative involving differences in the rate of fundamental metabolic reactions and in conditions associated with these is the only one that fits all of the facts at present known to us.

It would obviously be desirable, in order to establish the metabolic nature of the gradients beyond all reasonable doubt, to make quantitative tests of the rate of metabolism of different levels of the axis. To do so would appear to be a relatively simple matter, but in fact a number of complicating factors are involved. In the first place we must consider whether the isolation of pieces from different levels involves physiological changes in the pieces. We have definitely shown for *Planaria* that it does involve considerable metabolic increase, and that this increase is related quantitatively to the length of the pieces and the level of the body from which they are taken. To eliminate this factor it is necessary either to cut pieces so long that the stimulation of cutting is reduced to a minimum or to wait until the stimulation has disappeared, as happens after a number of hours. In the former case an organism of sufficient length would have to be selected; in the latter case one encounters changes due to the onset of regeneration. It is further necessary to select organisms which present neither structural nor special functional differences along their axes. The functional activity of some specific part may not accord with the general gradient, and

determinations of the total metabolism of such parts might therefore give results seemingly at variance with the gradient conception. The gradients of particular organs or structures are not necessarily the same as those of the organism in general; we know of many instances of such secondary gradients. Selection of the proper organism and elimination of these complicating factors are therefore essential before determinations of the total metabolism of isolated pieces possess any meaning.

On thinking over the organisms available for such experiments, it is obvious that they are few in number. Planarians are usable only under carefully controlled conditions. Under such control it has already been shown that the carbon-dioxide production at different levels of the anterior zooid corresponds with the gradient conception (Robbins and Child, '20), and similar experiments on the oxygen consumption are now in progress. Certain hydroids could probably be used, and experiments on them will be undertaken as soon as opportunity affords. The annelids are, however, by far the most suitable organisms for this kind of experimentation. Among the annelids there are many forms which are morphologically much the same throughout the greater part of the body in the sexually immature condition. They are, moreover, long enough to permit the cutting of pieces of the length necessary to eliminate the stimulation of section. As far as we have been able to determine, there are, in the non-sexual state, no special regions having a rate of activity different from that of the body in general. The susceptibility method has shown that the gradient of the digestive tract is the same as that of the body wall. By means of methylene blue the gradient of the nervous system has likewise been determined to be of the same character.² In these animals, then, there is every reason to predict that pieces cut from different levels of the body will show differences in respiratory rate which will accord with the susceptibility differ-

² In making a previous note on the use of methylene blue for demonstrating minor gradients (*Jour. Exp. Zool.*, vol. 24, p. 55), the senior author, being at that time ignorant of the fact, failed to credit Mr. John Wood MacArthur, formerly of this laboratory, with precedence in the use of this reagent, and takes this opportunity of correcting that omission.

ences. This prediction has been verified in the most satisfactory manner.

The experiments reported in this paper concern the rate of oxygen consumption per unit weight of pieces from anterior, middle and posterior levels of the polychaetes *Nereis virens* and *Nereis vexillosa*, and the oligochaete *Lumbriculus inconstans*. The experiments on *Nereis* were performed by the junior author at the Puget Sound Biological Station, Friday Harbor, Washington, in the summer of 1920. Those on *Lumbriculus* were performed by the senior author at the University of Chicago in the fall of 1920.

EXPERIMENTS ON LUMBRICULUS

1. *The susceptibility gradient of oligochaetes.* The susceptibility gradients of a number of oligochaetes including *Lumbriculus inconstans* were described in detail by the senior author in a former paper (Hyam, '16). It was found that in oligochaetes in general a double gradient is present—that is, the anterior and posterior ends are the most susceptible to toxic solutions and this susceptibility decreases toward the middle. The gradient in these forms may then be described as of the shape of the letter U, except that the two limbs of the U are of unequal length. In some species the anterior end is more susceptible than the posterior end, in which case the left-hand limb of the U would be longer, while in other species the posterior end is more susceptible, making the right-hand limb of the U longer.

In *Lumbriculus inconstans* the posterior end of the body is generally the most susceptible part. In some individuals the head is about equally susceptible, but usually is less so. The susceptibility decreases from both ends toward the middle of the worm, the least susceptible part being anterior to the middle, generally in about the second quarter of the worm's length. These relations are subject to considerable variation in different individuals. In the posterior part of the body there are further present in many individuals physiological indications of the existence of a second zooid. *Lumbriculus* reproduces asexually by autotomy. It may break in pieces at any level, but very commonly breaks

at a more or less definite level near the posterior end (see line x in fig. 2). When stimulated or placed under altered conditions, the worms are likely to snap off their posterior ends, which thereupon regenerate into complete individuals. The vast majority of worms taken in the field will be found regenerating their posterior ends. The place at which the fission occurs is in many individuals detectable by its increased susceptibility to toxic solutions. Whether it preexists before the worm is placed in the toxic solution or whether autotomy is initiated (but not completed or rendered visible) as a consequence of placing the worm in the solution is difficult to say. Regeneration experiments reported in the paper already referred to indicate that the region preexists. It should be emphasized that not all individuals give evidence of the existence of a breaking level.

In figure 1 are presented graphs of the death gradients of three individuals of *Lumbriculus inconstans* in solutions of potassium cyanide. In constructing such graphs the number of segments is plotted on the abscissa and the time of death on the ordinate. As the posterior segments are considerably shorter than the anterior ones the number of segments per unit of the cross-section paper has been increased at the posterior end. The anterior end of the animal is at the left, posterior end at the right.

The graphs illustrate the general character of the gradient in this species. This type of gradient is characteristic of annelids in general, of vertebrate embryos, and in all probability of all segmented animals, at least in embryonic stages. It is due to the method of growth of segmented animals by the formation of new segments in front of the anal segment. All three of the individuals show the presence of the zone of autotomy described above; in two of them this zone is well marked, while in the third it is only slightly in evidence.

This susceptibility gradient involves not only the body wall, but also the digestive tract and all visible systems. The transparency of this species enables one to see everything except the nephridia. Sex organs are completely wanting. If, then, the susceptibility gradient is in reality a metabolic gradient, it must follow that in this worm pieces cut from the posterior end must

consume the most oxygen, pieces from the anterior end less oxygen, and pieces cut slightly anterior to the middle the least oxygen. This was found to be the case.

2. *The electrical evidence.* To complete the evidence brief mention may be made of the electrical gradient and galvanotaxis of oligochaetes. It has been found that the anterior and posterior ends of oligochaetes are electronegative (galvanometrically)

Fig. 1 Graph of the death gradients in cyanide of three individuals of *Lumbriculus inconstans*. Segments on the abscissa; time of death in hours on the ordinate, reckoned from the beginning of death, not from time in the killing solution. Heads of animals to left; posterior ends to right. The high peak near the posterior ends of two of the individuals is due to the presence of a zone of autotomy.

to the middle, and further that when placed in an electric current the worms bend into a general U-shape, with anterior and posterior ends directed toward the cathode and middle region directed toward the anode.³ These facts further indicate that the anterior and posterior ends of oligochaetes have a higher metabolic rate than the middle regions.

³ Cf. further Hyman ('18). Near the top of the second column on page 523 in this paper the statement that oligochaetes in a current 'travel to the anode' should read 'travel to the cathode.'

3. *Material and method.* The species used, *Lumbriculus inconstans* Smith, was obtained from pools in the woods near the village of Clarke, Indiana. These pools, filled with fallen leaves and moss, are the characteristic habitat of this species. The pools dry up in summer or fall, depending on their size, and when this occurs the worms pass into an encysted state, emerging when the pools become filled with water again in the spring. The best time to collect the worms in numbers is when the pool has dried down until only a small puddle of water is left in the lowest part. In this puddle the worms collect in great numbers and may be picked out by spreading out the debris from the pool on a dry spot. The worms used in these experiments came from three collections made on October 1st, 12th, and 15th, and from two different pools. They were kept in the laboratory in dishes of well-water filled with debris from their native pools and were used within three weeks from the time of collection.

The worms used were 40 to 60 mm. long and consisted of 150 to 200 segments. As many worms will be found regenerating the tips of the tails it was not possible to avoid entirely using such worms. Worms showing signs of regeneration elsewhere than the tip of the tail were discarded. Three pieces were cut out of each worm, as illustrated in figure 2. The head of the worm was cut off and discarded and the first piece then cut immediately behind the head. The second piece was cut immediately posterior to the first one. The third piece was cut near the posterior end, the extreme posterior end, including the regenerating tip if such were present, being discarded. The first two pieces were of equal length, being usually 3 to 5 mm. long and containing ten to twelve segments. The posterior piece was generally cut longer than the other two pieces, owing to the smaller diameter of the posterior end, and was about 8 to 10 mm. long, containing twenty to thirty segments. There was naturally some variation in the length of the pieces corresponding to the variation in the length of the worms from which the pieces were cut. These pieces are too long to be stimulated by the cutting except of course at the cut surfaces, where such stimulation always occurs. However, as each piece possesses two cut surfaces, this factor is equalized.

It may be emphasized that these pieces are as nearly alike morphologically as is possible to obtain in elongated animals. The digestive tract of *Lumbriculus* is not differentiated except

Fig. 2 *Lumbriculus inconstans*, showing the manner of cutting the three pieces whose oxygen consumption is given in table 1. *x* indicates zone of autotomy.

Fig. 3 *Nereis virens*, showing the manner of cutting the three pieces whose oxygen consumption is given in tables 2 and 3.

in the head region, where it forms a pharynx; but this, as, already stated, is cut off and discarded. There are no reproductive organs. The circulatory system forms a loop with the sacs characteristic of the lumbriculids in each segment. The only differ-

ences concern the relative proportions and sizes of the parts of the animal at different levels. Even such differences are practically nil between the first two pieces, as the diameter of body and digestive tract and length of segments are much the same throughout the anterior half of the worm.

By reference to the graphs in figure 1, it will be seen that pieces 1 and 2 are cut from the descending part of the gradient and that piece 3 generally includes the zone of autotomy when present. The lowest part of the gradient of *Lumbriculus* falls between the thirtieth and sixtieth segments. In order to avoid with certainty the ascending gradient of the posterior two-thirds of the body, the second pieces were cut well in the anterior part of this region of lowest rate. It is of course impossible to know the details of the gradient in each individual worm, and hence considerable variation in the total oxygen consumption per unit weight in different sets of pieces may be expected. It is nevertheless certain that the oxygen consumption will be correlated with the levels from which the pieces are taken.

As *Lumbriculus* is rather small it was necessary to cut a considerable number of pieces for each experiment. A count of the number of pieces was not made, but the number was probably in the neighborhood of two hundred. All of the pieces from each level were placed in a 500 cc. Erlenmeyer flask. This was filled air tight with water, of which a sample was taken for analysis. The flasks containing the pieces were then allowed to stand at constant temperature for twenty-four hours. In some experiments the pieces were cut in the morning and the experiment run from the afternoon of one day to the same time of the following day. In other cases the pieces were prepared late in the afternoon and the experiment begun the next morning. The second procedure was planned to eliminate any stimulation due to section if such might be present, but gave the same general results as the first plan. After the twenty-four-hour interval a sample of water was drawn from each of the experimental flasks and its oxygen content determined. The oxygen content was analyzed by Winkler's method. Further details will be found in previous publications (Hyman, '19). The pieces of worms

were then collected in a funnel of filter-paper, dried on filter-paper, and weighed. The weights of such sets of pieces varied from 100 to 200 mgm. in various experiments. The sets of pieces from the three levels were of course tested simultaneously in each experiment and were handled in the same way throughout. There was some movement among the pieces, particularly at the beginning of the experiments. As far as could be ascertained the amount of movement did not differ in pieces of different level.

TABLE 1

Showing the rate of oxygen consumption of pieces from different levels of Lumbriculus inconstans, the pieces taken as illustrated in figure 2 and tested simultaneously. Temperatures ± 1

NUMBER OF EXPERIMENT	TEMPERATURE	PIECE 1 (ANTERIOR)	PIECE 2 (MIDDLE)	PIECE 3 (POSTERIOR)
		Cubic centimeters of oxygen consumed per gram per twenty-four hours		
	°C.			
1	19.5	2.53	2.35	2.89
2	19.5	4.35	3.70	4.46
3	19.0	3.93	3.74	4.35
4	19.0	3.98	3.83	5.79
5	18.0	Lost	4.21	4.81
6	17.5	3.25	2.70	4.66
7	19.5	5.71	3.34	6.58
8	19.5	4.21	3.32	7.07
9	19.0	4.35	3.52	6.26
10	19.0	4.99	3.81	6.86
11	19.0	5.15	4.45	6.19
12	19.0	3.01	4.11	7.76
13	19.0	5.17	3.32	5.17

4. *Experimental results.* Thirteen experiments were performed, the final results of which are presented in table 1. The results are calculated in terms of cubic centimeters of oxygen consumed by one gram in twenty-four hours.

In all of the experiments except no. 12, the anterior pieces consume more oxygen per unit weight per unit time than the middle pieces. The reversal of this result in experiment 12 may reasonably be regarded as due to some error in the course of the experiment, probably a mixing of the sample bottles. In all of

the experiments the posterior pieces consume more oxygen than the middle pieces and in the majority of cases they also consume more than the anterior pieces. In experiment 13 the oxygen consumption of the posterior pieces is the same as that of the anterior pieces, while in experiment 2 it is but slightly larger than the latter; but in most cases the posterior pieces have a markedly higher rate of respiration than the other pieces. The differences in the oxygen consumption in different experiments may be assigned to variations in the animals themselves.

The results on the oxygen consumption at different levels are therefore in complete accord with the susceptibility gradients, as shown in figure 1. The existence of metabolic gradient in this annelid may be regarded as demonstrated.

EXPERIMENTS ON NEREIS

1. *The susceptibility gradient of polychaetes.* Owing to the opacity and toughness of the majority of polychaetes, it has not been possible to apply the susceptibility method to them except in the case of the family Syllidae. In this family the worms are of small size and sufficiently delicate and transparent to permit observations on the death gradient. In the Syllid *Autolytus cornutus* the senior author found the gradient to be like that already described for oligochaetes (Hyman, '16, p. 118). There are further at hand the observations of Cwiklitzer, ('05) on the death gradient of *Ophyotrocha puerilis*, one of the Eunicidae. In order to study the regenerative capacity of this species, Cwiklitzer cut off very small portions of the head and noted in many of the operated individuals the occurrence of disintegration processes. These changes began first at the posterior end, then at the head, or at both anterior and posterior ends simultaneously, and proceeded from both ends toward the middle. In *Nereis* kept in the laboratory the senior author has noted in several cases the death of the posterior region, beginning at the anal segment and proceeding forward. These scattered observations indicate that the susceptibility gradient of polychaetes is similar to that of the oligochaetes.

2. *The electrical gradient.* The electrical gradient of freshly collected *Nereis* and its galvanotactic reaction are the same as those already described for oligochaetes. The anterior and posterior ends are electronegative (galvanometrically) to the middle region. When placed in an electric current, the worms bend into a U-form, anterior and posterior ends directed toward the cathode and middle toward the anode.

From these lines of evidence it may be expected that the oxygen consumption of different levels will vary in the manner already described for *Lumbriculus*. Posterior levels should consume the most oxygen, anterior less, and middle pieces the least. Experiment proves that such is the case.

3. *Material and method.* Two species were employed, *Nereis virens* Sars and *Nereis vexillosa* Grube. The former species was collected from the docks at the town of Friday Harbor; in such locations the worms are found among the *Mytilus*, tunicates, and similar forms which cover the piles. A few individuals of this species were also collected along the shores. The worms used were about 150 mm. long. *Nereis vexillosa* was obtained by digging in gravelly and sandy shores at low tide. The individuals used in the experiments were from 160 to 180 mm. in length. Sexually mature individuals were discarded. The worms were used as soon as possible after collection—from half an hour to three or four hours in nearly all cases. In two cases, experiments 6 and 7, table 3, the worms were kept about twenty hours before being utilized.

In preparing the worms for the experiments, the first 25 mm. and the last 10 to 15 mm. of the body were cut off and discarded. Three pieces were then cut from the body of the worm, short pieces being discarded between the first and second and second and third pieces. The way in which the pieces were taken from the worm is shown in figure 3. The pieces were 30 to 40 mm. in length. In each experiment several worms were employed, generally six or seven, once five, and twice twelve. After cutting, the pieces were placed in finger-bowls and washed in several changes of sea-water until bleeding had ceased and the cut ends contracted. All of the pieces of each level were placed to-

gether in a wide-mouthed bottle and their rate of oxygen consumption was then determined as already described for *Lumbriculus*. The tests ran from one to two hours. The pieces were then dried on filter-paper and weighed. Six or seven such pieces weigh from 2 to 10 grams; anterior and middle pieces weigh more than posterior pieces.

It may be pointed out that such pieces from different levels are nearly identical morphologically. The only differentiated part of the digestive tract in *Nereis* is at the anterior end, and this part of the body was discarded. The posterior end with its terminal cirri was likewise discarded. The only differences among such pieces are differences in the proportions of parts, as in the parapodia, which are largest in the middle regions of the body. The middle pieces probably have the advantage of greater surface of exposure, but, as will be seen, they nevertheless respire the least. It should also be stated that there was very little movement among these pieces.

4. *Experimental results.* The experiments on *Nereis virens* are recorded in table 2; those on *Nereis vexillosa* in table 3. Only the final calculations of the cubic centimeters of oxygen consumed per gram per hour are given. In the first two experiments on *Nereis virens*, the posterior pieces were not tested.

Examination of these tables shows that anterior and posterior pieces of *Nereis* invariably consume more oxygen per unit weight per unit time than middle pieces. Further, in nearly all cases the posterior pieces have a higher rate of oxygen consumption than the anterior pieces. There is one exception to this, experiment 5 in table 3, where the oxygen consumption of the posterior pieces is below that of the anterior pieces. In experiments 5 and 7, table 2, the posterior pieces are but slightly in advance of the anterior pieces, but in the other experiments the difference is marked. These variations in the experimental results are no doubt due to physiological differences in the individuals employed.

The existence of a metabolic gradient along the axis of *Nereis* may therefore be regarded as demonstrated. This gradient is identical with the susceptibility and electrical gradients.

TABLE 2

Showing the rate of oxygen consumption of pieces from different levels of Nereis virens, the pieces taken as illustrated in figure 3, and tested simultaneously

NUMBER OF EXPERIMENT	TEMPERATURE	PIECE 1 (ANTERIOR)	PIECE 2 (MIDDLE)	PIECE 3 (POSTERIOR)
		Cubic centimeters of oxygen consumed per gram per hour		
	°C.			
1	10.5	0.053	0.032	
2	10.0	0.042	0.035	
3	10.5	0.036	0.030	0.051
4	10.2	0.047	0.036	0.059
5	12.0	0.062	0.040	0.063
6	9.0	0.046	0.034	0.058
7	12.0	0.054	0.037	0.057
8	10.7	0.039	0.031	0.053
9	10.5	0.053	0.033	0.061

TABLE 3

Same for Nereis verillosa

NUMBER OF EXPERIMENT	TEMPERATURE	PIECE 1 (ANTERIOR)	PIECE 2 (MIDDLE)	PIECE 3 (POSTERIOR)
		Cubic centimeters of oxygen consumed per gram per hour		
	°C.			
1	9.0	0.061	0.039	0.079
2	9.5	0.040	0.034	0.055
3	10.0	0.047	0.033	Lost
4	12.5	0.078	0.060	0.094
5	10.0	0.062	0.038	0.050
6	13.0	0.057	0.043	0.083
7	13.0	0.040	0.035	0.074
8	10.0	0.059	0.041	0.081
9	10.5	0.074	0.058	0.089
10	9.5	0.041	0.036	0.063

SUMMARY

By means of the susceptibility and electrical methods, evidence has been accumulated which clearly indicates the existence of a double metabolic gradient in the oligochaete and polychaete annelids. According to this evidence, the anterior and posterior ends possess the highest metabolic rate, and from these ends the rate decreases toward the middle regions; in the majority of forms the posterior end has a higher metabolic rate than the anterior end.

In this paper are presented direct determinations of the rate of oxygen consumption per unit weight of pieces from anterior, middle, and posterior regions of the oligochaete *Lumbriculus inconstans* and the polychaetes *Nereis virens* and *Nereis vexillosa*. The results of these determinations are that the posterior pieces of these species consume the most oxygen per unit weight per unit time, the anterior pieces less, and the middle pieces least.

The results therefore confirm the conclusions drawn by the other methods and establish beyond reasonable doubt the existence of a metabolic gradient in these forms.

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