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RELATIONS BETWEEN METABOLISM AND MORPHOGENESIS DURING REGENERATION IN TUBIFEX TUBIFEX. II.

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Analysis of the relations between metabolism and morphogenesis requires that each set of processes be separated into component parts. Metabolism may be fractionated by means of agents of which the effects on particular enzyme systems are reasonably well known and the relation of the activity of such systems to morphogenesis may then be tested. In the annelid, *Tubifex tubifex,* morphogenesis during posterior regeneration may be measured fractionally and "rate of localization," "rate of early differentiation," and "rate of later differentiation" expressed quantitatively (Collier, 1947). It was found that oxygen consumption and loss of weight by starving worms proceed at a markedly increased rate during certain stages of regeneration, and that rate of oxygen consumption was correlated with "rate of later differentiation"; a metabolic (energetic) cost of differentiation was hypothesized and it was thought possible that this might be characterized by activity of particular enzyme systems. The present report concerns the effects of continuous poisoning by cyanide and by iodoacetate and also the effects of high oxygen tension, low oxygen tension, and complete lack of oxygen upon morphogenesis during posterior regeneration in the oligochaete annelid, *Tubifex tubifex* Mull.

Materials and Methods

The worms were handled and examined as described earlier (Collier, 1947). For high oxygen tension, gas from a tank was bubbled continuously through the water in which the worms were kept. Presence of a low percentage of carbon dioxide (about 5%) was found to have no effect on experimental results. For low oxygen tension, nitrogen or hydrogen was bubbled through the water at two-day intervals, the bottles being tightly closed between treatments. For strictly anaerobic conditions, hydrogen from a tank was first freed of traces of oxygen by passing it over platinized asbestos heated to a dull red; then it was bubbled continuously through wash bottles and experimental bottles in series. That the continual disturbance did not affect regeneration was ascertained by using a control set-up through which air was bubbled.

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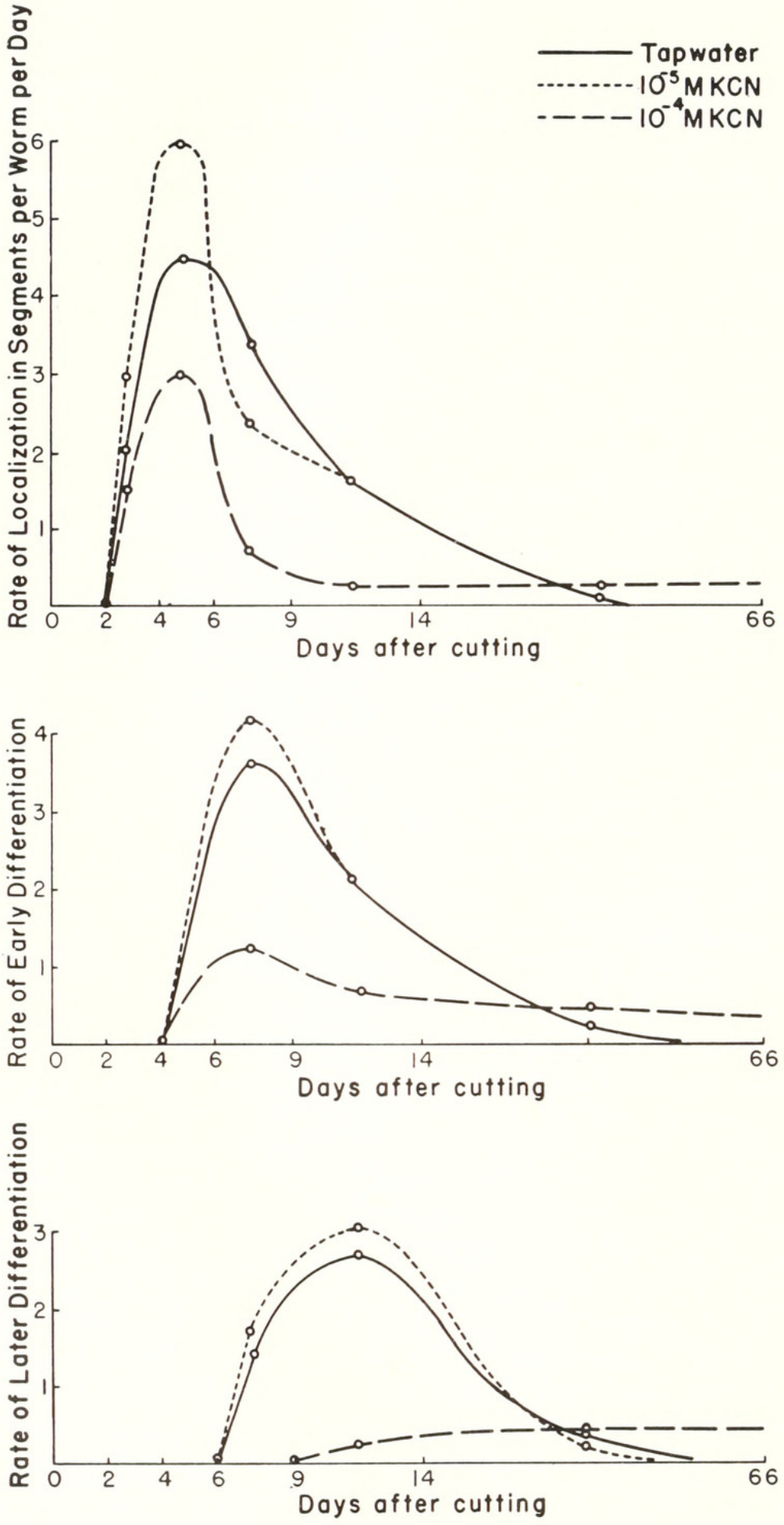

FIGURE 1. Effect of cyanide on rates of progress through various stages of regeneration. Mean deviation within each group of worms (thirty individuals) ranged from 0.3 to 0.5 segments per worm per day, increasing with time.

"Rate of localization" was calculated as increase in total number of segments per worm per day; "rate of early differentiation" as increase in number of segments showing some cellular differentiation visible *in vivo* under low power; and "rate of later differentiation" as increase in number of segments showing setae.

Effects of Cyanide on Regeneration

Over 300 worms were used in experiments involving continuous poisoning by potassium cyanide. A group of thirty worms in 10^{-3} *M* KCN became inactive and showed heavy mortality after the second day. The last survivors formed blastemae, but no localization of new segments occurred in nine days. Worms in 10^{-4} *M* KCN and 10^{-5} *M* KCN survived well, one group in 10^{-4} *M* showing 60 per cent survival at 159 days. A few individuals showed abnormal regeneration: one double tail and seven with the new tail at an angle. Since three of these eight were in a control group, the abnormalities could not be attributed to effects of cyanide.

Table I

Effect of potassium cyanide on progress of regeneration: segments per worm per day

Days	Rate of localization					Rate of early differentiation					Rate of later differentiation				
	2–4	4–7	7–10	10–15	15–94	2–4	4–7	7–10	10–15	15–94	2–4	4–7	7–10	10–15	15–94
Worms in 10^{-5} *M* KCN	4.7	2.4	1.4		0.2	0	2.0	2.2		0.3	0	0	1.4		0.4
	4.8	2.4				0	2.0				0	0			
to tap water			↘ 1.1		0.2			↘ 2.3		0.3			↘ 1.4		0.4
Worms in 10^{-4} *M* KCN	2.0	1.9	0.5		0.3	0	0	1.1		0.4	0	0	0		0.5
	2.4	1.7				0	0				0	0			
to tap water			↘ 1.3		0.3			↘ 2.0		0.4			↘ 0		0.5
Worms in tap water	3.0	3.0	1.5	→0.8	0.2	0	2.0	2.2	→1.4	0.3	0	0	1.3	→1.9	0.4
to 10^{-5} *M* KCN				↘ 1.3	0.2				↘ 1.7	0.3				↘ 2.1	0.4

Data on rates of localization, early differentiation, and later differentiation are summarized in Figure 1. During the first six days the worms in 10^{-5} *M* KCN showed a significantly higher rate of localization than controls, while worms in 10^{-4} *M* KCN showed a lower rate. Later the advantage of the worms in 10^{-5} *M* KCN disappeared, but the disadvantage of those in 10^{-4} *M* KCN continued. At 66 days the worms in 10^{-5} *M* KCN and in tap-water had practically completed regeneration: all new segments were in later stages of differentiation and had grown to almost the size of the old segments. The total number of new segments was almost precisely the same for these two groups (average 41 per worm). At this time the worms in 10^{-4} *M* KCN still showed segments in the early stages of formation and the average total number of new segments was only 26 per worm. Growth of the new segments was poor. But at 159 days their condition was fully comparable with that of the others at 66 days. The total extent and perfection of regeneration were unaffected by treatment, but rate of regeneration was markedly affected.

The areas under the rate curves at the top of Figure 1 represent the average total number of segments localized per worm. For tap water and 10^{-5} *M* KCN, these areas between two days and nine days were 23 and 25, respectively, or not sig-

nificantly different, despite the early rapid rate of localization in dilute cyanide. It may be presumed that the amount of cellular material available for localization of segments was a limiting factor, that this amount was unaffected by 10^{-5} *M* KCN and that the narrowness of the peak of rate was due to more rapid exhaustion of the material. The low rate of localization in worms in 10^{-4} *M* KCN and the fact that the area under the curve from two to nine days is less than half of that under the control curve suggests that availability of cellular material may have been decreased in 10^{-4} *M* KCN. The rapid decline to a very low rate of localization strengthens this suggestion.

Since rate of early differentiation during a particular interval of time should be limited by the number of segments localized, the differences in rates of differentiation were accounted for by the previous differences in production of localized segments. It appeared probable that the cyanide affected some process or processes occurring during or preceding localization, and had no direct effect upon later processes.

TABLE II

Regeneration in low oxygen

Days after cutting	Average number of new segments per worm		
	Nitrogen	Hydrogen	Control (air)
2 to 7	blastema	blastema	blastema
10	1.4	1.5	4.7
13	2.1	2.5	7.5 (setae)
16	3.7	3.0	9.8
19	5.0	5.1	11.7
23	6.0 (setae)		13.8
26	7.0		14.7

Mean deviation was about ± 1.0 segment up to 16 days, ± 2 thereafter.

An experiment was set up to test this idea. Five groups of thirty worms each were cut for regeneration and two groups were placed in 10^{-5} *M* KCN, two in 10^{-4} *M* KCN and one in tap water. At the end of seven days one group from each cyanide solution was transferred to tap water. Also, at the end of ten days the group in tap water was separated worm for worm into two comparable groups and one of these transferred to 10^{-5} *M* KCN. Data (summarized in Table I) in general confirm the supposition that cyanide affects localization and not later processes. Further, it is suggested that this effect is not upon mobilization of neoblasts, which, according to Krecker (1923) and Stone (1932), cease their metamorphosis and migration well before ten days. Rather the effect must be upon some process more directly concerned in localization.

EFFECTS OF LOW OXYGEN TENSION

If high oxygen tension acts as a stimulus to regeneration as suggested by Barth's work on Tubularia (1940), regeneration should be inhibited or retarded by low oxygen tension, while high oxygen tension should accelerate it. Exactly opposite

TABLE III

Observations on worms kept continuously under oxygen-free atmosphere

	18 hours	30 hours	42 hours
20 intact in tap water	dark red inactive	survival about 30%	survival 15%
20 intact in 10^{-5} *M* KCN	dark red inactive	survival about 80%	survival 45%
20 regenerating in 10^{-5} *M* KCN	dark red inactive	survival about 95%	survival 25%
20 intact in 10^{-6} *M* NaIAc	dark red inactive a few dead	no survivor	
Controls with air bubbled through tap water 20 intact 20 regenerating	normal	normal	survival 100%

results would be expected from the line of reasoning that in low oxygen tension there might be an increase in glutathione (*cf.* Barron, 1951) which, as found by Coldwater (1933), can increase rate of regeneration in Tubifex. One might then expect high oxygen tension to retard or inhibit regeneration.

One hundred and fifty worms were used in two experiments with atmospheres containing less than 4 per cent oxygen (Table II). No morphological abnormalities appeared, but there was a general retardation of regenerative processes.

Results of an experiment on survival of worms under more strictly anaerobic conditions (Table III) showed that this species cannot long endure complete absence of oxygen. Survival was significantly briefer in presence of iodoacetate.

TABLE IV

Effect of lack of oxygen on early stages of regeneration. (Each day access to air was permitted long enough to allow return of normal color and activity)

	2 days	4 days	5½ days	7 days
Oxygen-free Tap water	survival 7/30 1/7 with blastema	survival 0		
10^{-5} *M* KCN	surv. 30/30 24/30 with bl.	30/30 with small blast.	surv. 12/30 no blastema	survival 3/12
10^{-8} *M* NaIAc	no survivor			
Under air Tap water	30/30 with blastema			survival 30/30 aver. 19 new segments
10^{-5} *M* KCN	15/15 with blastema	aver. 11 new segments		survival 15/15
10^{-8} *M* NaIAc	15/15 with blastema			survival 15/15

Controls gave assurance that no materials from the apparatus or wash solutions had been responsible for destruction of the experimental worms.

For the purposes of studying regeneration an experiment was set up in which survival was improved by allowing a short period of access to air once a day (Table IV). The worms did produce blastemae at the usual time but regeneration proceeded no further and the blastemae disappeared. It appears that dilute cyanide improved survival but in the absence of oxygen did not show its accelerating action on localization.

The effect of anaerobiosis on later stages of regeneration was tested using worms which had regenerated for seven days under normal conditions (Table V). The worms kept under oxygen-free atmosphere (except for twenty minutes at 27 hours) showed practically no progress in regeneration, while in the control an average of 3.6 new segments per worm had been localized, 5.7 had undergone early differentiation, and 7.1 later differentiation. Regeneration here requires the presence of oxygen.

TABLE V

Effect of lack of oxygen on later stages of regeneration

	0 hours	47 hours
15 worms under oxygen-free atmosphere		survival 100%
Aver. no. segments in localization	7.0	6.2
Aver. no. segments in early differentiation	11.2	10.0
Aver. no. segments in later differentiation	0.6	1.7
Aver. no. segments total per worm	18.8	17.9
15 worms allowed to continue under air (Control)		survival 100%
Aver. no. segments in localization	7.0	4.8
Aver. no. segments in early differentiation	11.3	9.9
Aver. no. segments in later differentiation	0.6	7.7
Aver. no. segments total per worm	18.9	22.5

Mean deviation was about $\pm$ 1.0 segment.

Effects of High Oxygen Tension

Concurrent with the experiments with low oxygen, two groups of thirty worms each were kept under an atmosphere of 95% oxygen and 5% carbon dioxide and a third group under 100% oxygen. Results were the same in all groups. The worms survived well for several days and formed blastemae, but regeneration proceeded no further and all worms had died by ten days. To test whether inhibition of localization by oxygen and stimulation of localization by 10^{-5} *M* KCN might be based on opposite effects on the same mechanism, experiments were set up in which worms were kept in 10^{-5} *M* KCN, 10^{-4} *M* KCN, and 10^{-3} *M* KCN under an atmosphere of pure oxygen (Table VI). The 10^{-5} *M* KCN partially counteracted the effects of oxygen both upon survival and upon regeneration; 10^{-4} *M* KCN was found to partially counteract the effect of high oxygen upon regeneration, but it did not even partially counteract the lethal effect. Accordingly, high oxygen tension had an effect on regeneration independently of its lethal action and, far from stimulating regeneration in Tubifex, very high oxygen inhibits it.

Effects of Iodoacetate

Since experiments in which worms were subjected to low oxygen tension and to complete lack of oxygen had suggested that glycolysis might be important for

TABLE VI

Simultaneous effects of high oxygen tension and cyanide on survival (in fractions) and regeneration (in average number of new segments per worm)

	2 days	4 days	6 days	9 days	14 days
Under oxygen Tap water	30/30 sm. blastema	23/30 0 seg.	4/30 0 seg.	no survivor	
10^{-5} *M* KCN	30/30 sm. blastema	30/30 2 seg.	28/30 3 seg.	21/30 4 seg.	no survivor
10^{-4} *M* KCN	30/30 sm. blastema	29/30 1 seg.	no survivor		
10^{-3} *M* KCN	no survivor				
Under air Tap water	30/30 blastema	28/30 4 seg.	28/30 13 seg.	27/30 23 seg.	27/30 31 seg.
10^{-5} *M* KCN	18/18 blastema	18/18 6 seg.	18/18 18 seg.	18/18 25 seg.	18/18 33 seg.
10^{-4} *M* KCN	15/15 blastema	12/15 3 seg.	10/15 9 seg.	10/15 11 seg.	10/15 12 seg.
10^{-3} *M* KCN	29/30 no blastema	no survivor			

survival and regeneration, worms were allowed to regenerate in various concentrations of sodium iodoacetate: 10^{-3} *M*, 10^{-4} *M*, 10^{-5} *M*, 10^{-6} *M*, 10^{-7} *M*, and 10^{-8} *M* NaIAc. Five intact worms in 5×10^{-3} *M* NaIAc showed decreased activity after six hours, and were dead at thirty-six hours. In the other concentrations intact worms survived as well as but no better than the regenerating worms. The group of thirty worms in 10^{-3} *M* solution showed high mortality after three days, but the few survivors maintained normal morphogenesis. The worms in 10^{-4} *M* solution showed high mortality after six days, but six of the thirty worms survived for twenty-four days and maintained normal regeneration. Mortality in the other groups was low. Rates of progress through various stages of regeneration were very nearly the same for all concentrations of iodoacetate (Fig. 2), but whereas rate of later differentiation in tap water reached a peak between thirteen and seventeen days after cutting, the rate of later differentiation in each of the iodoacetate solutions

TABLE VII

Effect of dilute iodoacetate upon rate of oxygen consumption

Sample	Oxygen consumption in cubic millimeters per milligram of worms (wet weight) per hour	
Large worms (over 4.5 cm.)	0.12 in tap water	0.13 in 2×10^{-8} *M* NaIAc
Small worms (under 2.5 cm.)	0.14 in tap water	0.13 in 2×10^{-6} *M* NaIAc

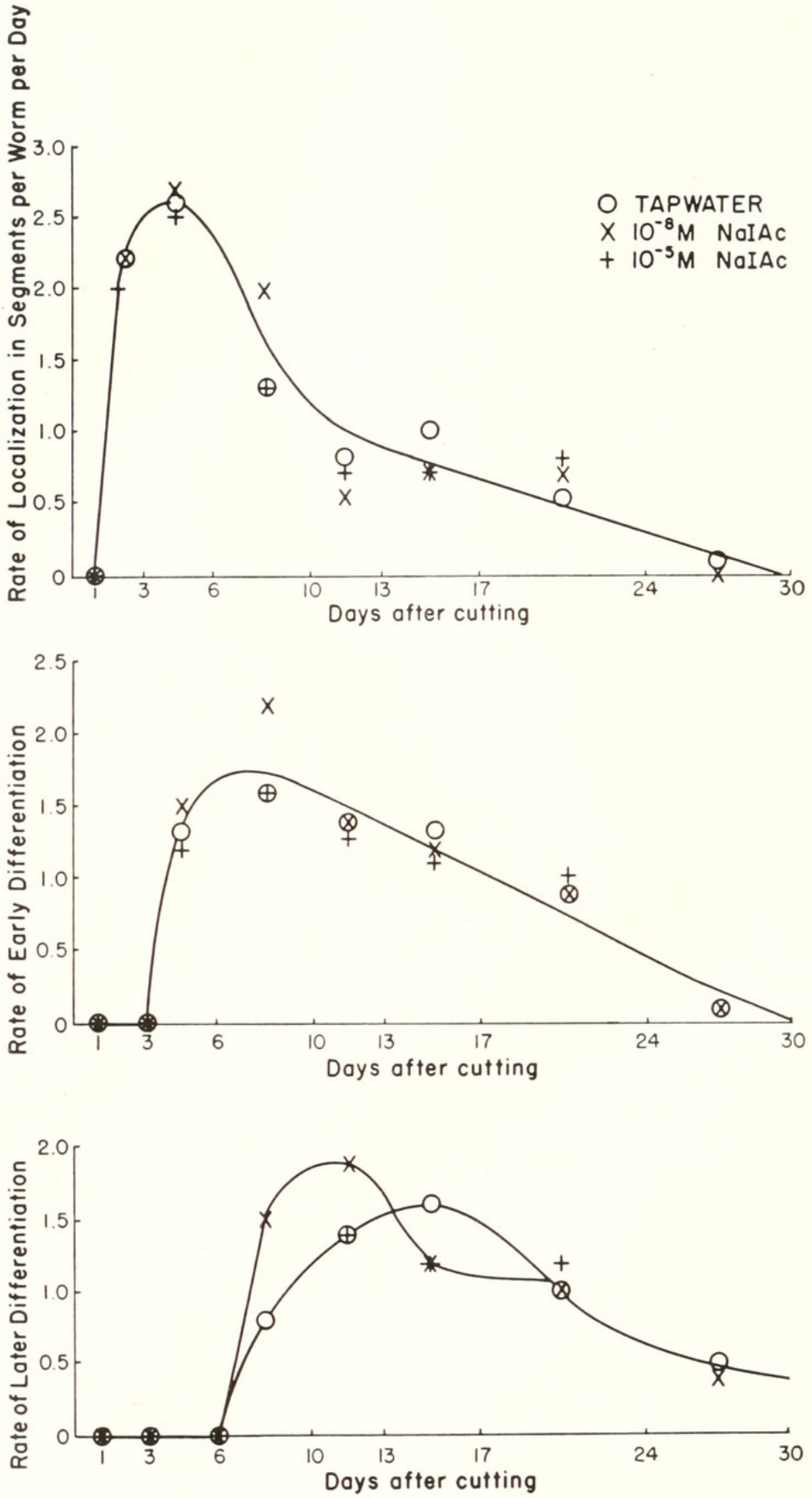

FIGURE 2. Effect of iodoacetate on rates of progress through various stages of regeneration.

reached its peak between ten and thirteen days after cutting. Iodoacetate appeared to accelerate later differentiation without affecting earlier processes. Because of the range of concentrations used, and because the extremely dilute solutions used here had maximal effect on differentiation, it may be presumed that iodoacetate poisons some process(es), inhibition of which allows further activity of some other process(es) in a system of multiple pathways of hydrogen and electron transfer (*cf.* Lipmann, 1954). The inhibited process might be glycolysis, while the reciprocally related process might or might not be concerned in the increased oxygen consumption previously found during the period of most rapid "rate of later differentiation."

A determination of the effect of sodium iodoacetate upon oxygen consumption of normal worms was made (Table VII). It is clear that iodoacetate had no significant effect on rate of oxygen consumption.

Discussion

It has been held that differences in rate of metabolism in the various parts of an animal may be the basis for production of morphological differences (Child, 1940; Hyman, 1940; Barth, 1938, 1940). Much of the supporting evidence comes from experiments on regeneration of hydroids, and the extremely rapid rate of regeneration here makes it difficult to distinguish between a factor influencing initiation of regeneration and one limiting later processes. In Tubifex, slow regeneration permits sufficient time for more detailed analysis. Since regeneration here is initiated even in the complete absence of oxygen, increased oxygen tension in the tissues at the cut surface is obviously not the primary stimulus nor is it even a necessary condition. Instead the availability of oxygen acts as a limiting factor in the progress of certain later processes in regeneration. However, the concept may be applied to morphogenesis during regeneration in Tubifex when used as Lindahl (1936) applied it in the echinoderm egg: differences in rate of particular fractions of metabolism may be the basis for certain initial processes in morphogenesis.

In the experiment in which worms with partially regenerated tails were subjected to lack of oxygen, the metabolism which supported vital processes did not support morphogenetic processes. Accordingly, regeneration must depend upon activity of some aerobic pathway. The fact that cyanide affected "rate of localization" indicates a cyanide-sensitive system important during localization. The fact that cyanide did not affect rate, extent or perfection of differentiation indicates that the particular system has little or no importance in relation to differentiation. Accordingly, on the basis of cyanide-sensitivity the processes supporting "localization" and "early differentiation" are distinct. Similarly, on the basis of sensitivity to iodoacetate the metabolic processes of "early differentiation" and of "later differentiation" are distinct.

During the period in regeneration before "later differentiation" appears, oxygen consumption was found to be only slightly, if at all, above normal (Collier, 1947). However, these worms lost weight almost twice as rapidly as controls, and this suggested an energetic cost of localization which was not reflected in oxygen consumption. The same applied to a possible cost of "early differentiation," but "later differentiation" was found associated with a markedly increased consumption of oxygen. Determination of respiratory sensitivity to cyanide showed that the increase was cyanide-stable. This contrasts with the findings of Bodine and Boell

(1934) for grasshoppers and of Sanborn and Williams (1950) for Cecropia moths, that the additional oxygen consumption during development is cyanide-sensitive although the respiration during diapause is entirely cyanide-stable. In fact it appears that the metabolic mechanisms of morphogenesis in metamorphosing insects (*cf.* Williams, 1951) can hardly be compared with those in regenerating Tubifex.

The presence of oxygen was found to be essential to "localization" but high oxygen tension inhibited it. There is no necessity for assuming that normal oxygen tension should establish optimal conditions for localization. Since these worms normally live partly submerged in mud, the optimum might be an oxygen tension lower than that established in very shallow mudless tap water under air. Fox and Taylor (1955) found this true for survival and growth of young worms in the laboratory.

Respiration as measured by the Warburg method was entirely stable to 10^{-4} *M* and 10^{-5} *M* KCN, but continuous exposure to these concentrations of cyanide affected "rate of localization." 10^{-4} *M* KCN was found to retard while 10^{-5} *M* accelerated "localization." Both concentrations counteracted the inhibitory effect of high oxygen tension.

It was considered that the accelerating effect of the more dilute cyanide solution is comparable to the often observed and seldom explained stimulation of various processes by other inhibitors in extreme dilution (*cf.* Commoner, 1940). Since in other cases the stimulation is effective upon the same processes which are inhibited by higher concentrations of the poisons, it was considered that the two concentrations of cyanide affected the same process in "localization." The concentrations of cyanide which activate proteinases *in vitro* are at least fifty times higher than 10^{-4} *M*, and were rapidly lethal to the worms (more minutely described by Hyman, 1916). Nevertheless cyanide here may have been effective upon the reactions of some metalloprotein other than those of the cytochrome system or of the haemoglobin in the blood of these worms. The antagonism of high oxygen damage by cyanide does suggest that the effects of high oxygen tension and of cyanide do meet somewhere, but if we assume that cyanide here is acting as an oxidative poison, then the cyanide-sensitive system cannot be responsible for any large proportion of the oxygen consumption: it may be off the main electron transfer pathway. The lethality of high oxygen tension also suggests an autoxidizable system that is off the main pathway (Gerschmann *et al.*, 1954). Whatever high oxygen affects, whether protein synthesis, concentration of particular normal or abnormal components, structural integrity, etc., it was at least partially counteracted in Tubifex by cyanide.

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Summary

1. Continuous exposure of regenerating *Tubifex tubifex,* Mull. to cyanide has been found to affect "rate of localization" without affecting the ultimate extent or perfection of localization or of other morphogenetic processes.

2. Continuous exposure to iodoacetate has been found to increase "rate of later differentiation" without having other effects on regeneration.

3. Low oxygen tension was found to retard regenerative processes generally. In complete absence of oxygen, blastema formation took place but all subsequent processes were effectively blocked.

4. High oxygen tension blocked morphogenesis and also was lethal in from four to eight days. Both the inhibition and the lethal effects were partially relieved by concurrent treatment with cyanide.

5. It is concluded that the availability of oxygen limits the progress of later processes in morphogenesis without playing any necessary part in the initiation of regeneration in Tubifex.

6. It is indicated that metabolic processes supporting "localization," "early differentiation," and "later differentiation" are at least partially distinct from each other and from the metabolic processes essential to maintenance; that energy released in the promotion of particular morphogenetic processes must be released through particular enzyme systems; and that such specific release of energy is essential to the progress of morphogenesis.

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