

Formic Acid-Method for Clarifying *in toto* the Intestinal Aspect of the Planarian

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ABSTRACT It has been desired to provide the intestinal tracts easily visible in planarian regeneration. From the purpose, a new method was established that the procedure is as simple and plain as any because the worm is only treated by formic acid, except the comments that the damageable specimen due to acid treatment is held in safety and sealing the worm between both glasses must be completely kept in order to avoid the evaporation of formic acid. By this method, several aspects of the intestinal tracts can be clearly recognized. Thus, detailed aspect of normal intestinal system were described. An attention should be, however, paid that this method could be applied to the starved worm but not to the fed one. If the worm as fixed with various ordinary fixatives after treatment with formic acid, clearance of the intestinal tracts disappeared. Acetic and lactic acids were endowed with also efficiency for clearing the intestinal tracts though formic acid was more advantageous than them. From these observations, formic acid-method was discussed in some view points.

Introduction

The triclad flatworms have been classical material for studies on regeneration. Regeneration of the intestine was especially interested with what this organ is significant in concerning that it occupies the greater part of the body and branches into three main tracts which are characteristic representation of this animal. It was, however, entirely difficult to exactly secure an aspect of the intricated intestinal tracts in the worm by means of routine method followed in the serial paraffin sections. It must be, therefore, convenient for studies on intestinal regeneration to establish such method as the intestinal tracts are brought in clearly visible condition with unequivocal way. Although the whole-mount preparation has been used for observation of the intestine of intact planarian, this method was unable to be used in starved animal.

Before about a decade, the authour had found the fact that the planarian intestinal tracts can be clearly recognized by treating the worm with formic acid, and then the method have been applied expedientially to observe the normal or regenerating planarian

intestine in our laboratory though the reason which form basis of this phenomenon could not be understood (Kido 1961, Kido and Kishida 1961). The present report aim at demonstrating the unpublished method for clarifying in toto the intestinal system of the planarian under the starved condition and at considering this formic acid-activity.

Material and Methods

The Planarians used were exclusively *Dugesia japonica* collected in vicinity of Kanazawa city. Normal well-developed planarians were selected and starved for ten days or more and then were treated with following procedures. Besides, regenerating worms and several worms transplanted the head piece into their postpharyngeal region were also examined.

(A) Procedure for formic acid-method.

- 1) Place a worm in one drop of tap water on the slide glass.
- 2) Pour one drop of 80% formic acid¹⁾ (full-strength of commercial reagent) by the pipette with rubber nipple when the worm elongated himself.
- 3) Cover the worm with the deck glass which is supported with two fine glass-rods in equal thickness to that of the worm body.
- 4) Pour 20% formic acid (diluted into quadruple of 80% commercial reagent with distilled water) gently into the space between the slide glass and the deck glass.
- 5) After flowed solution was wiped off thoroughly by the blotting paper,²⁾ seal the deck glass with the melted paraffin by means of the heated steel spatula. It is recommended that the slit along the contact lines between slide glass or deck glass and settled paraffin are coated with fingernail lacquer (Nail Polish) by a single brushing in order to take away completely evaporation of formic acid.
- 6) After several minute, observe and photograph with low power of microscope or print directly on the sheet-film by means of the photographic enlarger.

(B) The examination of the activity of formic acid.

- 1) The influence of some chemical fixatives on the formic acid-activity.

On the test whether or not particular clearance of planarian intestinal tracts following formic acid-method is able to be preserved by treatment of the chemical fixatives, above procedure (A) was in turn succeeded up to step 4), and in next step formic acid was soaked up with the blotting paper from one side of the deck glass, simultaneously the various fixatives were poured there from the opposite side. Fixatives used were as follows; Nozawa's, Gilson's, Smith's, Regaud's, Flemming's, Carnoy's, Bouin's fluids, 10% formalin and absolute alcohol.

On the other hand, the worms fixed previously with the same fixatives were treated with formic acid.

- 2) Treatment with acetic acid and lactic acid.

In order to learn whether the other organic acids than formic acid are effective for clarifying of intestinal tracts, the worms were treated with acetic

acid or lactic acid. The procedure was the same with the formic acid-method(A), but concentrations of acetic acid used were 50%, 25% and 10%, and lactic acids used were solutions diluted with distilled water to 1/2 or 1/4 of the syrup of commercial reagent.

Notes

- 1) Concentration of formic acid may be diluted up to 40% depending on the planarian batch used.

Since formic acid, even its vapour, is very harmful for human skin, man needs to be cautious of treatment of this acid. If concentrated acid attached to your skin, you must wash it *at once*.

- 2) Whenever a little flowed solution remains, sealing become incomplete because the melted paraffin floats on it, and consequently the mounted specimen will be destroyed on account of bubbles arising from evaporation of formic acid.

Results and Observation

(A) Formic acid-method.

When a drop of formic acid was poured on a worm he dead with elongating himself, and the brown body pigment dissolved out. After several minutes the intestinal tracts were distinctly realized with their minutes detail under the microscope because of the result that the intestine only remained in opaque and the other parts became transparent (Fig. 1). In this case, it is noteworthy that visible parts due to the opaque in the intestine are their wall but not their lumen, so that single intestinal tract was seen as what consisted of two opaque lines and one translucent line. (Fig 6).

From observation with this method, the normal intestinal system can be subdivided into four parts as follows. (Fig. 2).

- (1) Main tracts: canal system which consists of single tract running from head to pharyngeal base in prepharyngeal region and two tracts running from the pharyngeal base to tail tip in pharyngeal and postpharyngeal regions.
- (2) Branches: canals which directly branched out of the main tracts.
- (3) Twigs: small canals which diverged from each branch.
- (4) Acini: blunt ends of each twig.

In prepharyngeal region, number of the branches in each side was about fifteen. Two main tracts in pharyngeal and postpharyngeal regions, which may be regarded as branches divided from the main tract anterior to the pharynx, branches the many twigs on the outside but had only short rudimentary branches without twigs on the inside. In some cases, the acini in prepharyngeal region anastomosed with the adjacent twigs or acini to make network or spontaneous lateral canals. Array of the intestinal tracts in head region was standardized considerably in most specimens, while it was conspicuously diverse in trunk and tail regions. The head, in most cases, had a single main tract running between the eyes and two or three branches running in side of the eye. (Fig. 2). It

was shown frequently that some opposite branches in postpharyngeal region fused each other on the median line of the body (Fig. 6).

The clearance of the pharynx and copulatory organs could not be so distinct as the intestine, but only their situation and gross aspect could be recognized (Figs. 4 and 5).

In contrary to the body pigment, eye pigment was not dissolved by formic acid, so that the eye of the worm could be observed very clearly after applying formic acid-treatment (Figs. 1 and 2).

While intestinal tracts of formic acid-treated animals which were observed under the microscope were relieved in dark against light background, they were seen in light against dark background if reflected light was used for the dissective microscope under which the black paper was placed (Fig. 12). In latter way, every discretion to take off even any tiny dusts must be required as contamination in the preparation was highlighted troublesomely. Stereo-construction of the intestinal tracts frequently appeared more distinctly in the latter way than in the former.

When the worm fed with the calf liver was treated with formic acid, the intestinal array was undistinguishable though the body was transparent (Fig. 7). In regenerating worms from prepharyngeal or postpharyngeal pieces, old intestine in stem and regenerated one were clearly distinguished one another even after 20 days (Figs. 9 and 10). In the worms which were induced new pharynges by transplantation of head into postpharyngeal region, connection between intestines of host worm and graft was revealed distinctly (Fig. 11 and 12).

If the sealing of the deck glass was complete, the material could be preserved and examined at least for a year. If strong (concentrated) formic acid was used in mounting, epithelium of the worms tented to stripped off and come to fragments.

(B) Analysis as to advantage of formic acid-method.

When the worms which were treated with formic acid to bring about clearance of intestinal tracts were immersed in non-alcoholic fixatives as formalin, Smith's, Regaud's, Flemming's and Bouin's mixtures, their transparent bodies became turbid as a whole and then their intestinal tracts turned out to be not seen. On the other hand, the clear images of the intestinal tracts disappeared gradually from their acini as the worm body remains in transparence by treatment with some alcoholic fixatives as Nozawa's, Gilson's, Carnoy's mixtures and absolute ethanol (Fig. 8)

When acetic acid and lactic acid were used in place of formic acid, the worm became transparent to bring out apparency of the intestinal tracts though its rate was slower than with formic acid and its degree was not reached to one with formic acid since both acids did not extract the body pigment.

Discussion

Hitherto, large numbers of histological observations have been undertaken in studies on planarian regeneration. But, in spite of many efforts, some conflictions were remained on account of the fact that histological events occuring in planarian regeneration were

too complex to be ascertained (Brøndsted 1969). In order to resolve these questions, it needs to know as morphological whole what morphogenetic changes in the regenerating organs are progressing, though necessity that histochemical, autoradiographic or electron microscopical methods must be introduced in the study of this line can be justly considered. In planarians, methods for staining *in toto* have been developed on nervous system by Ooba (unpublished) using the Schiff reagent, and by Betchaku (1960) using silver staining, but the reports regarding to the intestine are few, as far as the writer is aware. Although the whole-mount preparation of the planarians which were fed with carmine powder mixed in boiled yolk of hen's egg (Tsuchida) or with carbon powder infiltrated in chick spleen (Ichikawa and Kawakatsu 1961) was suitable for clarifying the planarian intestine, these methods were unable to apply to the regenerated pieces, because the worms must be starved customarily for some days before experiments and the worms cannot feed in early stages of regeneration. Formic acid-method described in the present paper was favorable in respect that it can clarify the intestine even in the starved and regenerating worms and is very simple and plain, that is, only mounting by diluted formic acid is offered without such troublesome course as fixing, dehydrating and clearing by solvents etc.

It was discerned by formic acid-method that the planarian intestine consists of four components, i.e. main tracts, branches, twigs and acini, and topographical aspect of these components was revealed without any misperception. The observation by the present method may contribute to future studies on intestinal regeneration. The intestine which incorporated the foods could not become visible by formic acid. This phenomenon is probably ascribed to distending the intestine and to intestinal cell-lysis by the cause occurring after feeding as suggested by Hauser (1956).

The fact that the intestinal sight clarified with formic acid-method was faded away by alcohol-treatment is significant for explaining the formic acid-activity: it is reasonable that most tissues in the planarian became transparent by formic acid except the intestinal cells included particularly with any alcohol-insoluble granules.

In this examination, the intestinal system not only became visible by formic acid but by the other organic acids such as acetic or lactic acids. It has been well known in histological examinations that the nuclei of muscle and the other tissues became visible by acetic acid-treatment. Transparency of the planarian body by acetic acid or lactic acid may be based on general character of organic acid.

The fact that formic acid cannot dissolve the eye pigment in contrast with body pigment is interest, suggesting that there is chemical difference between the both pigments; the former may be melanin-like pigment which is indissoluble by this acid and the latter is unknown pigment different from melanin. The author has revealed in previous papers that eye pigment disappeared by treatment with thiocarbamide and dithiocarbamide but the body pigment did not. (Kishida 1959, 1960a, b, 1961). This phenomenon should be also understood from the view point that chemical difference exists between the body and the eye pigments. The elucidation as to this will be discussed in the other paper.

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PLATE I

Plate I

The photographs represent the planarian bodies treated with formic acid, except fig. 3.

Fig. 1 Whole body of the planarian : Intestinal system and eyes are clearly seen. The preparation was printed directly on the electron-microscopic film (Fuji). $\times 6$

Fig. 2 Prepharyngeal region : It is shown that the main tract (m) consists of many branches (b), ramified twigs (t) and acini (a). $\times 14$

Fig. 3 Head region in the whole mount-preparation of the worm fed with the beef liver, where carbon powder was infiltrated. $\times 18$

Fig. 4 Pharyngeal region : Inner cavity (ic) is seen faintly. $\times 27$

Fig. 5 Copulatory organ (c) : $\times 27$

Fig. 6 Postpharyngeal region : Two main tracts and inner rudimentary (ib) and outer well-developed branches (ob) are clearly seen. A fused inner branches is seen (arrow).

Fig. 7 Prepharyngeal region in the worm fed with the beef liver : Intestinal tracts expand as clear as distinguishable by formic acid-treatment. main tract : mt ph : pharynx $\times 27$

PLATE I

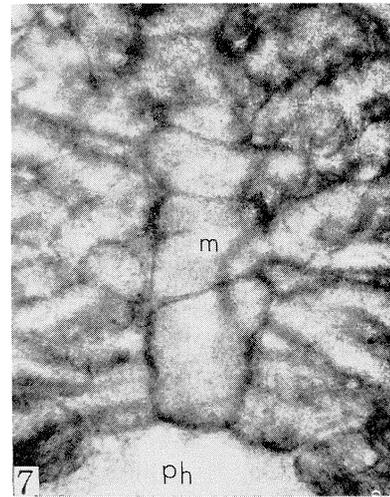
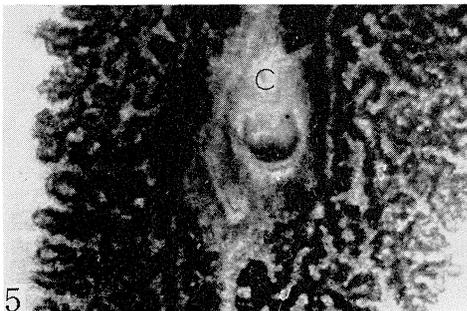
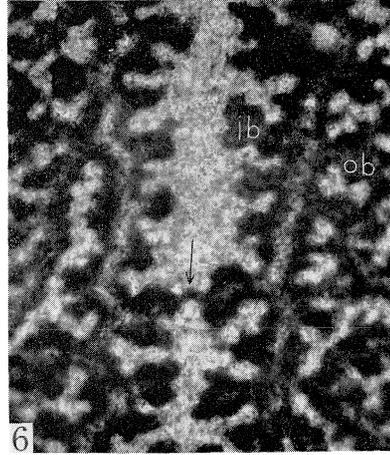
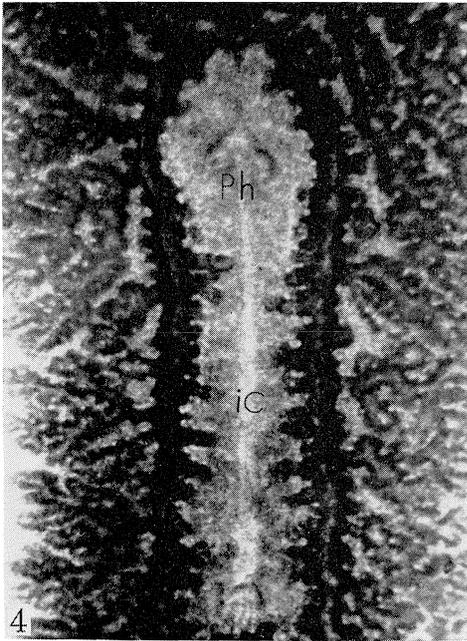
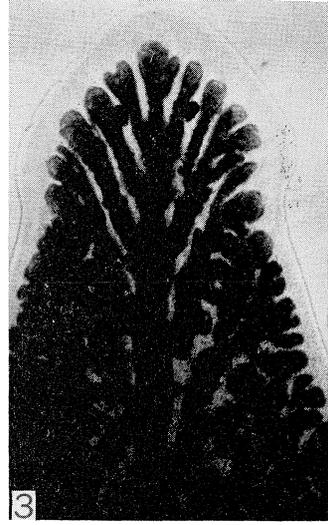
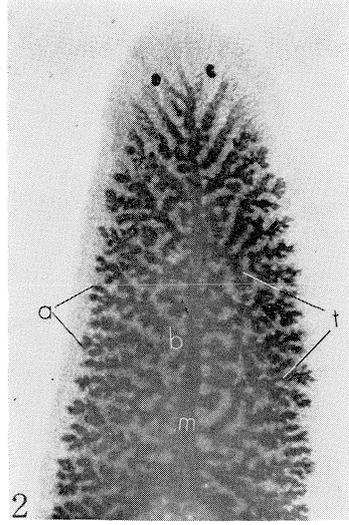
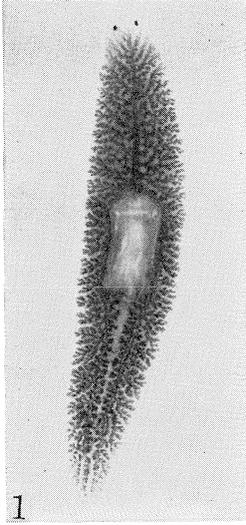


PLATE II

Plate II

The photographs represent the planarian bodies treated with formic acid.

Fig. 8 A planarian treated with formic acid after immersing in alcohol for one hour : Clear aspect of intestinal system fade away gradually from the margin. $\times 14$

Fig. 9 A worm regenerated from anterior cut surface in the postpharyngeal piece : Newly regenerated intestine in head region is brightly seen. $\times 20$

Fig. 10 A worm regenerated from posterior cut surface in prepharyngeal piece : Regenerated pharynx and two main tracts are clearly observed. $\times 20$

Fig. 11 A worm on the 20th day after transplanting the head piece into postpharyngeal region : The graft occurs in outgrowth (G) and induces two new pharynges (nph). $\times 25$

Fig 12 A worm on the 27th days after transplanting the head piece into postpharyngeal region : Photograph is taken in reflected light uuder dissective microscopes, Intestinal system are brightly seen against dark background. G : outgrowth of the graft nph : new pharynges induced $\times 27$

