IN VITRO CULTURE OF EMBRYOS IN THE SILKWORM, *BOMBYX MORI* L.

I. CULTURE IN SILKWORM EGG EXTRACT, WITH SPECIAL REFERENCE TO SOME CHARACTERISTICS OF THE DIAPAUSING EGG

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(With Plate 9)

It is generally believed that the diapausing egg of the silkworm contains a substance which is transmitted from the mother and has an inhibitory action upon the development of the embryo. Watanabe (1924) was the first author to explain the phenomenon of diapause in the silkworm egg by postulating this substance which he called 'inhibitory substance', and almost all subsequent authors in Japan have accepted this assumption. It is not unreasonable to suppose that the 'inhibitory substance', which reveals its action in the egg after deposition, has some connexion with the diapause factor (hormone) secreted by the suboesophageal ganglion of the mother (Hasegawa, 1951, 1952; Fukuda, 1951), but nothing is known about the real nature and action of the diapause factor (Fukuda, 1955).

On the other hand, Umeya (1937*a*, *b*, 1938, 1939, 1952) insists that in the diapausing egg, although the embryo is always ready to grow, some unfavourable condition of the yolk surrounding the embryo inhibits its development beyond a certain stage during diapause, while Miura (1932*a*, *b*, 1938) claims that not only the yolk, but also the embryo itself remains inactive in the diapausing egg. The author has investigated the culture of the silkworm embryo *in vitro* with the object of elucidating these problems by separating embryos from their native yolk and culturing them with experimental media. This paper deals with the results of culturing embryos with silkworm egg extracts.

MATERIAL

Diapausing, non-diapausing, acid-treated, chilled, and chilled-and-acid-treated eggs of various silkworm strains were used as materials.

Diapausing eggs. In diapausing eggs, yolk cells begin to migrate towards the periphery of the egg about 2 days after deposition, and with the completion of this movement in 2 weeks or so (Takami, 1953, 1954*a*) the eggs reach a resting stage (Nittono, 1955; Nittono & Takeshita, 1953). This stage is cited as full diapause in this paper. Such a fully diapausing egg, whose embryo is shown in Pl. 9, fig. 3,

cannot be activated until it has over-wintered. Watanabe (1931) reported that diapausing eggs, if kept at 25–27° C. continuously, usually died after about 190 days, only very few eggs being able to hatch. It is probable that activation of the eggs is not checked at these temperatures, but is retarded so long that most of the eggs die before the completion of activation (Kutsukake, 1954). The eggs used in the present work were widely different in age, ranging from 1 day to several months at 25° C., with corresponding differences in the degree of diapause. Embryos of 1-day-old diapausing eggs were at the broad germ-band stage (Pl. 9, fig. 1).

Non-diapausing eggs. Non-diapausing eggs are also at the broad germ-band stage 20–24 hr. after deposition, and reach the beginning of appendage formation at about the 40th hr. at 25° C. In the present study, eggs 20–30 hr. old were used.

Acid-treated eggs. Diapausing eggs at about the 20th hr. can easily be changed to non-diapausing eggs artificially by immersing in hydrochloric acid (specific gravity 1·075, 46° C., 5 min.). Eggs thus treated were rinsed in running water for about 10 min. and used for experiments before appendage formation.

Chilled eggs. These were eggs which had been stored at 5° C. from the 20th or 50th hr. for different periods until use. The eggs are sufficiently activated to start growth at the end of about 90 days' cold storage, showing good hatchability without any acid treatment if incubated at 25° C.

Chilled-and-acid-treated eggs. One month's cold storage at 5° C. from 50 hr. after deposition is usually insufficient to activate diapausing eggs completely, but during cold storage the eggs become sensitive to acid treatment, and can be changed to non-diapausing ones by a supplementary treatment with somewhat stronger hydrochloric acid (specific gravity 1·10, 47° C., 5 min.) than in the case of the above mentioned acid-treated eggs. The eggs thus treated are called chilled-and-acid-treated eggs in this paper.

METHOD

It is convenient to use silkworm eggs laid on paper. Pieces of a suitable size for handling were cut out from the egg paper bearing the eggs, washed in 5% formalin for 5 min., rinsed in distilled water, and after immersion in 94% ethyl alcohol (two changes of 2–3 min. each) sufficiently dried to ensure adhesion of the eggs. The fixing of eggs on the paper is desirable to facilitate subsequent operations. After the drying, the chorion of the egg was carefully cut open with a sharp needle under a dissecting microscope, and the piece of egg paper transferred to salt solution (Table 1) in a watch-glass in which embryos were dissected out of the eggs with needles and pipettes. An embryo thus obtained was freed from yolk cells adhering on it by repeated gentle sucking in and out of a small-bore pipette, if necessary with additional use of needles, and then put on the underside of a cover-slip by means of the pipette. The salt solution which accompanied the embryo to the cover-slip was replaced with a drop of the culture medium described below. The cover-slip was sealed on a depression slide with melted paraffin, making a hanging-drop culture which was incubated at 25° C. It was necessary to add sufficient culture medium to cover the explanted embryo, because the explant cultured with

insufficient medium always swelled and died without showing any sign of development. All these procedures after immersion in alcohol were carried out under aseptic conditions.

Salt solutions and culture media. The object of the present work was to find out if there is any difference between diapausing and non-diapausing embryos in their growth response to the yolk. By the 'yolk' is meant the silkworm-egg contents which can be sucked up with a small-bore pipette, so that it may contain mostly yolk together with other egg inclusions. The eggs supplying yolk were used after the same treatments, washing in formalin, immersion in alcohol and sufficient drying, etc., as those which supplied the embryos. The response of the embryos to each component of the yolk is an ultimate object of this study, but not the problem of this paper.

Table 1. *The salt solutions used for dissecting eggs and preparing culture media*

	Solution 1	Solution 2
(A) NaCl	7·5 g.	7·5 g.
KCl	0·5 g.	0·5 g.
$CaCl_2.2H_2O$	0·2 g.	0·2 g.
$NaH_2PO_4.2H_2O$	0·05 g.	0·05 g.
Glucose	—	0·2 g.
Distilled water	750 ml.	750 ml.
(B) $NaHCO_3$	0·05 g.	0·05 g.
Distilled water	250 ml.	250 ml.

A and B are sterilized separately, and mixed in this ratio after cooling. pH 6·7–7·0.

An extract of eggs, used as the culture medium, was made by mixing the yolk (1 part) thus obtained with the salt solution (2 parts) presented in Table 1. In the earlier experiments this mixture was allowed to settle for several minutes after stirring, without being centrifuged, and a comparatively clear part of it was sucked up as the extract for culturing embryos. It was, however, inevitable that the egg extract thus prepared often contained (in addition to abundant suspended particles which would gradually settle on the explant) embryonic fragments which were able to grow and lead to confused development (Pl. 9, fig. 6). The later experiments, therefore, were carried out after eliminating these materials by centrifuging the extract in a glass-tube (2·5 × 30 mm.) for 10 min. at 1000 r.p.m. (the extract from diapausing eggs older than 3 days) or at 1500 r.p.m. (the other extracts). By such centrifugation the extract was fractionated into three layers, that is, a sharply limited upper, lower and rather clear middle layer. The last occupied the greater part of the tube, showing a gradual change in clearness from one end to the other, and could be used as a suitable culture medium. In the diapausing egg, as a result of the migration of yolk cells, the aqueous phase begins to be segregated from the non-aqueous phase about 3 days after deposition. This becomes more distinct with age, and, accompanying this change, fractionation becomes easier in the older eggs. It was for this reason that the fractionation was carried out at different speeds of

rotation according to the age of the eggs. Since the results of cultures with the centrifuged extract showed the same tendency as those obtained with the non-centrifuged extract so far as the growth response of embryos was concerned, most of the experiments with the former will be presented below, together with some supplementary data from cultures with the latter.

Salt solution 1 may be somewhat hypotonic, causing swelling of embryos. However, even these swollen embryos were able to develop in the extract prepared with this solution, and when they started development in the extract their normal shape was restored within a day. In solution 2, swelling of embryos was not observed. Though the two solutions differed from each other in the inclusion of glucose, the two kinds of egg extracts prepared with these solutions showed similar effects on culturing embryos, apart from the swelling of embryos in solution 1. Physiological solutions with a higher ratio of potassium to sodium were recommended for lepidopterous insects, in the course of the work, by many authors (Barsa 1954, Ishikawa & Miyoshi, 1956, Wyatt, 1956); but since the author had started the experiment with the solutions described, the formula was kept unchanged throughout the series.

Embryos never grew in the salt solutions without addition of the yolk, though the change in shape from the broad germ-band to the slender embryo (Pl. 9, fig. 2) was observed in solution 2 as well as in the yolk extract.

Observation. Cultures were continued, without renewal of the medium, usually for 7–10 days during which development of the explants was examined with a low-power microscope every day, and at the end, examinations were made in detail after rinsing the embryos in the salt solution to remove adhering sediment and, if necessary, tearing away the amnion.

In many cases it is rather difficult to judge whether diapausing embryos in culture have really started development or not. For the sake of accuracy, therefore, appendage formation was taken as the criterion of development in the present work, because this was a development stage (Pl. 9, fig. 4) which was never reached by normal diapausing embryos. The following descriptions are based on about 300 and 500 cultures tested in 1955 and 1956, respectively.

RESULTS

Non-diapausing and fully diapausing embryos. When cultures were made with the extract from non-diapausing eggs, explanted embryos of non-diapausing eggs showed a noticeable difference in development from those of fully diapausing ones (Table 2). In the former, segmentation of the ectoderm, formation of the appendages and invagination of the stomodaeum and of the proctodaeum, etc., proceeded until the stage just before the revolution, bristle formation and mandibular pigmentation being often reached (Pl. 9, fig. 5). In the latter, the development, if started, was only partial, very rarely going on as far as appendage formation. Most of the thirty-six cultures of fully diapausing embryos shown in Table 2 resulted in swelling (a sign of failure in development) within a few days, and none reached appendage formation.

In cultures in which appendage formation of embryos was reached marked spreading of the amnion (Pl. 9, fig. 7) and migrating out of mesoderm cells were common, often being accompanied with hernia of the proctodaeum.

In cultures made with the extract from fully diapausing eggs, even non-diapausing embryos, which grow actively in the extract from non-diapausing eggs as mentioned above, showed a markedly restricted development, only 36·4% (8/22) of them reaching appendage formation. All the fully diapausing embryos which were tested failed to develop in this extract.

Table 2. In vitro *culture of non-diapausing and fully diapausing embryos with the egg extract from non-diapausing eggs* (*solution* 2)

Embryo	Non-diapausing			Fully diapausing	
	Over-wintered	Chilled	Acid treated	More than 1-month-old	25-day-old
Egg extract	Over-wintered, non-centrifuging	Chilled, non-centrifuging	Acid treated, centrifuging	Chilled, non-centrifuging	Acid-treated, centrifuging
No. of cultures	6	25	20	25	11
Appendage formation No.	5	25	18	0	0
Appendage formation %	94·1			0	

Table 3. In vitro *culture of* 1–2 *day diapausing embryos* (*solution* 2, *centrifuged*)

Embryo	1-day diapausing			2-day diapausing		
Egg extract	1-day diapausing	2-day diapausing	Acid treated	1-day diapausing	2-day diapausing	Acid treated
No. of cultures	32	5	23	11	3	16
Appendage formation No.	32	5	19	10	3	16
Appendage formation %	93·3			96·7		

Younger diapausing embryos. The behaviour of younger diapausing embryos *in vitro*, however, was not the same as that of the above-mentioned fully diapausing ones. Thus 1–2-day embryos developed well in the extracts from both diapausing and non-diapausing eggs of the same age as the embryos (Table 3). The culturing operation must therefore be an effective stimulus in activating the diapausing embryos which can seldom be activated *in vivo* by acid treatment. Diapausing embryos more than 2 days old became gradually less active with age in *in vitro* development (as seen in Table 4), 12-day embryos finally showing almost no growth. The final age, however, is different according to diapausing nature of explanted embryos.

In cultures made with the extract from diapausing eggs before the pigmentation of the serosa, conspicuous purplish red granules were observed on the developing embryos and amnion. It seems, from the results of experiments using many kinds

Table 4. In vitro *growth of diapausing embryos in relation to their age (five cultures each, solution 2, 1-day yolk, centrifuged)*

Age of explants (day)	Distribution of days until appendage formation					Total
	2	3	4	5	7	
1	1	4	—	—	—	5
2	2	2	—	—	—	4
4	—	1	1	1	—	3*
8	—	—	3	1	—	4
12	—	—	—	—	—	0

* Two cultures were discarded owing to bacterial infection.

Table 5. *Possible recovery in nutritive effect of the yolk in diapausing eggs kept at 25° C. continuously*

	Extract from eggs		
	20–40-day-old	50–90-day-old	140-day-old
No. of cultures	56	14	32
Appendage formation No.	19	4	22
Appendage formation %	33·9	28·6	68·8

1–2-day embryos were explanted.

of egg-colour mutants as material, that development of explants in media which contain 3-hydroxykynurenine is a necessary condition for the occurrence of these granules in cultures. This phenomenon recalls Horikawa's investigation on the pigmentation of eye disks of *Drosophila* in *in vitro* cultures with kynurenine-containing media (Horikawa, 1956), and will be dealt with in detail elsewhere.

Age of the yolk. The effect of the age of the yolk on culturing embryos is shown in Table 5. It seems that the further the diapausing eggs age at 25° C. the less suitable their yolk becomes for *in vitro* culture of embryos. This change in the yolk is parallel to the morphological and physiological changes in the yolk cells *in vivo* (Takami, 1953, 1954*a*). An interesting reverse change may possibly take place in the yolk after about 100 days' storage of the eggs at 25° C. The yolk recovers to a large extent the ability to nourish non-diapausing embryos *in vitro*, though little morphological change was noticed in the yolk cells at this time and the embryos in the same eggs had not yet recovered from diapause, showing neither hatch at 25° C. nor development *in vitro*. This recovery will need confirmation by further experiments.

Cold storage of eggs. The diapause of both the embryo and the yolk described above can be checked by cold storage of eggs. In Table 6, it is seen that the embryo and the yolk have been kept active for 20 days by keeping the eggs at 5° C. Cold storage for less than 20 days also gave similar results.

Table 6. *Cold storage at* 5° C. *of* 1–2*-day diapausing eggs (solution* 2, *centrifuged)*

Embryos from eggs	Extract from eggs	No. of cultures	Appendage formation
Stored at 5° C. for 20 days	Stored at 5° C. for 20 days	10	9
	Kept at 25° C. for 20 days	10	0
Kept at 25° C. for 20 days	Cold storage for 20 days	10	0
	Kept at 25° C. for 20 days	5	0

There is some reason to believe that the diapause of eggs may proceed to some extent even at 5° C., but in the present investigation, it could not be proved, probably because it is obscured by a strong activating effect of the explanting operation.

Heating of the egg. The non-centrifuged extract from the 2-day diapausing eggs which had previously been immersed in hot water at 60° C. for 5 min. was nearly the same in nutritive effect as the extract from non-heated eggs. Similar tests were also made with the extract from other heated eggs (75° C., a fews seconds) and these gave similar results, though heating of the centrifuged egg extract at 65° C. for 20 min. caused a considerable, but not complete, inhibition of the growth of explants. Further experiments, however, are desirable on this point.

DISCUSSION

The diapause of the silkworm egg is a maternally inherited characteristic and cannot, as a rule, be changed directly by the sperm entering the egg (Watanabe, 1924). It is evident that the diapause hormone is produced in the mother's body (Hasegawa, 1951, 1952; Fukuda, 1951) and affects the egg during the stay in the ovary. Therefore, the 'inhibitory substance' postulated by Watanabe (1924), if present, probably exists in the egg before embryo formation. On these grounds, it is likely that the diapause of the embryo is primarily determined by some condition of the yolk. Umeya's opinion (1937*a*, *b*, 1938, 1939, 1952) that diapausing embryos are always ready to develop, being inhibited only by some unfavourable condition of the yolk surrounding them, seems to be less plausible, because, as shown in Table 2, fully diapausing embryos could rarely be activated in *in vitro* culture with the egg extract suitable for the development of non-diapausing embryos. Younger diapausing embryos of the silkworm, or even a part of them, can be activated *in vitro* without any organic connexion (see below) with the yolk cells, in conformity to the Bucklin's result in *Melanoplus differentialis* (Bucklin, 1953). According to Bucklin small yolk-free fragments of the diapausing embryo explanted to hanging drops of Ringer's solution resumed development, indicating that the treatment exerts its effects directly on the individual tissues of the embryo, without the mediation of extra-embryonic structures or of any discrete endocrine organ. In this connexion, the possible recovery in nutritive effect of the yolk after more than 100 days' storage at 25° C. must be important, for it shows that the yolk may be

activated independently of the activation of the embryo, which still remains dormant in the same egg.

One-day-old diapausing eggs are highly sensitive to hydrochloric acid, all the eggs treated with this acid being changed to non-diapausing ones, while 3-day eggs are far less sensitive to the same treatment and fewer than 10% of the eggs can be activated (Watanabe, 1935). This change may be a manifestation of increasing dormancy, and can be explained in two ways: (1) the 'inhibitory substance' may have increased in quantity; or (2) some changes in the physiological processes concerned in diapause have progressed so far that they cannot be reversed. According to the first explanation, the termination of diapause necessitates consumption of the inhibitory substance under the effect of low temperature (Muroga, 1951).

The 'inhibitory substance', however, may not necessarily exist continuously in diapausing eggs; it may be present for a short time only, acting at a very early stage of embryonic development, and this may be sufficient for the determination of diapause. But no decisive results on this point have been obtained by the present experiments at the germ-band stage, owing to activation of the embryos by explantation alone and the possible absence of the 'inhibitory substance' at this stage. The activation of operated embryos has also been noticed by Yamazaki (1938) in wheat and by Bucklin (1953) in *Melanoplus*.

To test whether the yolk itself, in addition to the embryo, is also activated by the operation, preliminary experiments were carried out in which cultures were made with the extract from previously heated eggs. But more experiments are needed on this point. Schmidt & Williams (1953) have shown that the growth-promoting hormone contained in the active blood of the *Platysamia cecropia* silkworm could withstand heating at 75° C. for 15 min. It has recently been reported that addition of the extract from acid-treated eggs noticeably improved *in vitro* culture of the ovarian tissue in the silkworm, *Bombyx mori* (Wyatt, 1956), though whether the extract contains any hormone or not must be determined by further experiments.

The yolk of diapausing eggs is markedly different morphologically and physicochemically from that of non-diapausing eggs, so it is likely that, apart from such specific substances as the 'inhibitory substance' and the growth-promoting hormone, there is also some difference between these yolks affecting the availability of their nutritive components. This is a problem that could be made clear by means of *in vitro* culture.

The above results obtained in the silkworm are similar, in some respects, to those obtained by Yamazaki (1952) in wheat. He found that the endosperm of non-resting seed accelerates germination of the embryo transplanted into it, and that growth of the active embryo from non-resting seed cannot be inhibited by transplantation into the inactive endosperm of resting seed. He explained these phenomena by postulating the existence of some germination-promoting substance in both the embryo and endosperm of the non-resting seed, and of a germination-inhibiting substance in those of the resting seed. In contrast to this it has been proved by Schmidt & Williams (1953) that spermatogonia and spermatocytes from the diapausing pupae of *Platysamia cecropia* and *Samia walkeri* can be cultured

successfully up to the spermatid stage *in vitro* with the active blood from non-diapausing individuals. These results show that post-embryonic diapause is probably different physiologically from embryonic diapause.

The yolk of the silkworm egg is not merely a mass of non-living nutritive material, but is found in the egg as an inclusion of yolk cells. The yolk cell, or yolk spherule, is a living unit containing some yolk nuclei derived from cleavage nuclei in addition to a large quantity of yolk material, and can move independently (Takami, 1954*b*). These yolk cells keep an organic connexion with the embryo *in vivo*, it is possible therefore, that any change in the yolk will be reflected in the embryo and vice versa. In fact, detailed investigations have shown that the diapause of the silkworm egg is, strictly speaking, not a maternally inherited character, but is affected directly, to some extent, by the spermatozoon fertilizing the egg (Fujiwara & Ōyanagi, 1956; Kutsukake & Kuroiwa, 1951). In this sense, the possible recovery in nutritive effect of the yolk, independently of, or preceding, embryonic activation, is interesting in relation to Umeya's opinion that activation of the silkworm egg takes place in the yolk first, and that as a result of it the embryo becomes active secondarily. However, it has been proved by the author, contrary to his expectation, that the embryo is dormant in the diapausing egg. To make these points clear, experiments are now in progress to see whether the embryo free from its native yolk, and the yolk separated from the embryo, can be activated independently.

SUMMARY

1. Experiments have been carried out on the *in vitro* culture of silkworm embryos with silkworm egg extracts.

2. Non-diapausing embryos can be cultured well beyond the stage of appendage formation with the extract from non-diapausing eggs, while fully diapausing embryos at about the 30th day are, for the most part, unable to grow in the same extract.

3. 1–2-day-old diapausing embryos still retain the ability to develop *in vitro* as well as non-diapausing ones.

4. The extract from 1–2-day-old diapausing eggs has nearly the same nutritive effect on the *in vitro* culture of embryos as that from non-diapausing eggs, though the extract becomes less nutritive with age of eggs.

5. Diapause of both embryo and yolk can almost be prevented by cold-storage at 5° C.

6. The yolk of diapausing eggs kept continuously at 25° C. possibly recovers, after about 150 days, its nutritive effect, which falls with the onset of diapause. This change is independent of activation of the embryo, and is accompanied by little morphological change in the yolk cells.

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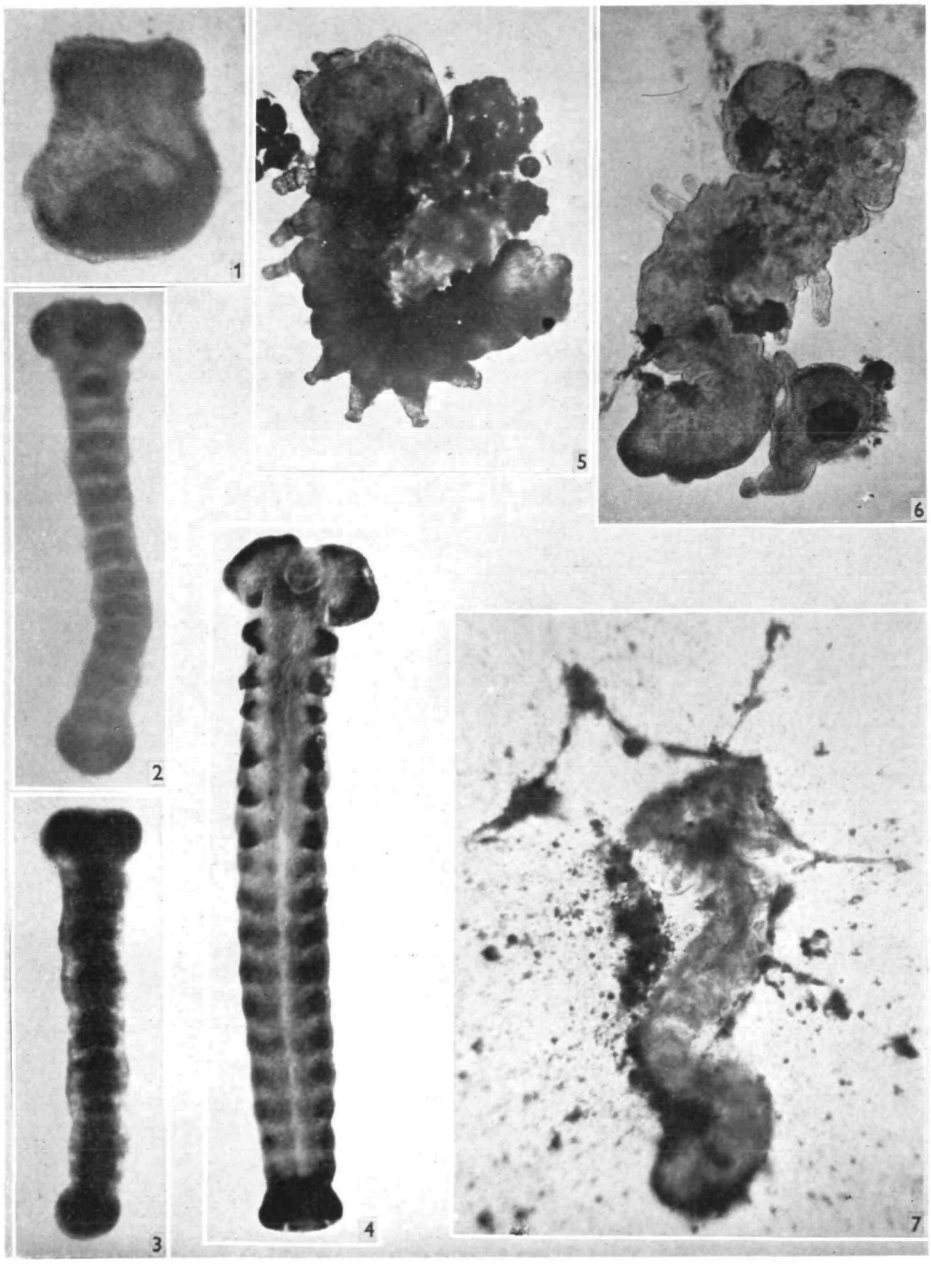
EXPLANATION OF PLATE 9

Fig. 1. A 2 hr. old germ-band.
Fig. 2. A 20-day-old diapausing embryo.
Fig. 3. A 50-day-old (fully diapausing) embryo.
Fig. 4. An embryo at the appendage formation stage.
Fig. 5. *In vitro* growth of a 2-day-old diapausing embryo (after 12-days' culture).
Fig. 6. *In vitro* growth of an acid-treated embryo with a developing embryonic fragment contained in the medium.
Fig. 7. *In vitro* growth of an embryo with spreading-out of the amnion.



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(*Facing p. 296*)