Reports

Identification of the Volatile Factor Involved in Spermatocyte Differentiation in vitro

Abstract. Ketchel and Williams postulated a "volatile factor" which participates in the differentiation of insect spermatocytes in vitro. It was found not to be a specific product of developing cysts. Instead, its effects are duplicated by a number of reagents which have only a high water content in common. The hypothesis that the volatile factor is water was experimentally confirmed.

Ketchel and Williams (*1*) have analyzed the properties of a volatile factor participating in the in vitro differentiation of insect spermatocytes into spermatozoa. This factor is of particular interest since, in the words of these authors, "the reaction between cells and hormones can generate molecules which are catalytically active and prerequisite for the biological end-result." Such an unusual and fundamental finding deserves confirmation and further examination.

Evidence for the volatile factor was based on the failure of unsealed depression slides to support differentiation and the requirement of several cultures in the same chamber when larger volumes were employed. The properties ascribed to the volatile factor, aside from its volatility, were that it was produced by the developing cells, was required by them for maximal differentiation in culture, and was eliminated from cultures by ventilation. It was insoluble in Ringer's solution, in dilute acids, in alkaline solutions, and in sucrose solutions, but was "soluble" in insect blood and "partially soluble" in mineral oil. It was absorbed by charcoal. By inference the volatile factor was neither a protein nor any other macromolecule; it was neutral, apolar, and not a normal constituent of air, such as carbon dioxide.

To identify the volatile factor we have investigated the differentiation of spermatocytes in hanging-drop cultures. The results differ from those of Ketchel and Williams (*1*), particularly with respect to the requirement for a threshold concentration of developing cysts for the production of the factor. Our results demonstrate that the volatile factor involved in spermatocyte differentiation is simply water.

Nondeveloping spermatocytes from diapausing pupae of the giant silk moth (*Samia cynthia, Philosamia cynthia* Drury, or *Samia walkeri*) were transferred to hanging-drop cultures according to the method of Schneiderman *et al.* (*2*). Blood was collected under aseptic conditions from abdominal incisions, as described by Laufer (*3*). "Inactive" blood, lacking the "growth and differentiation hormone" (presumably ecdysone, but possibly something else) was obtained from diapausing pupae. "Active" blood was obtained from individuals during the early phases of development into the adult. Blood from these animals permits spermatocyte development presumably because of its higher concentration of the "growth and differentiation hormone" (*4*).

Germinal cysts for culture were teased from testes and then checked microscopically for generally healthy appearance, sufficient concentration (a minimum of several hundred large cysts from each testis), and lack of development. The criteria for development after 5 days were those of Schneiderman *et al.* (*2*).

The volume of the culture chamber was varied by using alternatively, depression slides or two sizes of petri dishes (15 by 60 or 100 mm). Test drops containing active blood and cysts were placed on the inside of the cover, while additional drops of liquid were added either alongside the test drop or to the bottom of the dish. Each dish was sealed with Vaseline and incubated at 21° to 22°C. The number of drops of liquid as well as the nature of the liquid and its contents were varied to determine the effects of quality and quantity of fluid required for maximal differentiation of cysts under culture conditions.

The results of Ketchel and Williams (*1*) were corroborated to the following extent. (i) Single-drop depression-slide cultures of spermatocytes in active blood differentiated. An average of 42 percent of the cysts developed by 5 days (Table 1, ser. 1*a*). When a chamber was incompletely sealed, development was depressed or lacking; the culture fluid darkened and evaporated and the cysts dissociated and finally disintegrated. (ii) In larger chambers—that is, 60- or 100-mm petri dishes—single drops usually did not show signs of differentiation, while two or more (as many as four in the 60-mm dishes) yielded an intermediate percentage (0 to 12) of differentiating cysts. When a larger number of active cultures was placed in the same chamber, the percentage of differentiating cysts increased to maximal (control) levels (these values ranged between 35 and 52 percent for 6 to 16 drops; see Table 1, ser. 1*b*).

The "volatile factor" of Ketchel and Williams (*1*) was postulated mainly on the basis of observations of maximal cyst differentiation in a large chamber only when the chamber contained sufficient numbers of additional cultures also undergoing development. Thus, 16 drops of developing cultures were required to produce maximal differentiation. According to these authors, single cultures in 17-cm^3 containers resulted in 30 percent of the development seen when 16 cultures were present. Still only about 30 percent of the normal development was observed when a single drop of test cysts was cultured in the presence of 15 drops of active or diapausing blood without cysts or when 15 drops of cysts were cultured with diapausing blood alone. These additional drops did not contain developing cysts and therefore could not elaborate the volatile factor.

We repeated these experiments but obtained different results. In our experience, developing cysts were not necessary (Table 1, ser. 2). The culture chamber seems to become saturated with "volatile factor" whenever cultures of active blood and cysts are supplemented with additional drops of insect blood. It was found that there was no appreciable difference when test drops of active blood and cysts were cultured in the presence of "inactive" blood, of "active" blood without cysts, or of "inactive" blood with cysts. The feature that did appear to be significant was

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Type manuscripts double-spaced and submit one ribbon copy and one carbon copy.

Limit the report proper to the equivalent of 1200 words. This space includes that occupied by illustrative material as well as by the references and notes.

Limit illustrative material to *one* 2-column figure (that is, a figure whose width equals two columns of text) or to *one* 2-column table or to *two* 1-column illustrations, which may consist of two figures or two tables or one of each.

For further details see "Suggestions to Contributors" [*Science* **125**, 16 (1957)].

that a threshold number of drops of solution had to be present. Whether or not the inducing drops contained developing cysts made no difference in the result.

Additional experiments (Table 1, ser. 1*c* and 3) were performed to determine whether water could be substituted for "inactive" or "active" insect blood. Indeed, it was found that an equivalent amount of water could fully duplicate the effect of blood (ser. 1*c* and 3). Even in large petri dishes (ser. 3), with an estimated volume of 100 cm^3, the cysts in a single "active" drop differentiated when an excess of water was introduced into the bottom of the chamber. The cysts developed as well as control cultures in depression slides (ser. 1*a*). These experiments indicate that water can substitute fully for "active" cultures.

If water and the "volatile factor" are two independent growth factors involved in spermatocyte differentiation, then their effects should be additive, or at least they should produce significantly enhanced percentages of differentiation even at the saturation point for one of them, when the other factor is added for assay. Thus, if the two components act independently, eight drops of active cultures with an excess of water should give an appreciably higher percentage of cyst differentiation than a test culture with one drop of "active" blood and cysts with an excess of water. This expectation was not fulfilled (Table 1, ser. 4). Since either water or "active" cultures will substitute completely for the other in about the same quantity (ser. 1, *b* and *c*) and since there is no additive effect, we are forced to conclude that the "volatile factor" is, undoubtedly, water.

As additional confirmation the effects of desiccants were assessed. Culture dishes with sufficient numbers of active drops to permit maximal development were compared with similar dishes to which the desiccant was added. The additive was either indicating silica gel (mesh size, 6–16), which is capable of absorbing more than 40 percent of its weight in water, or copper sulfate. Control cultures contained 0.1 to 0.2 g of the water-saturated desiccant, while the experimental cultures were given the same material after it had been dried in heat.

The cultures with dry desiccant produced a lower percentage of developing cysts than those with the saturated reagent (Table 1, ser. 5). While we do not know whether silica gel or copper sulfate absorbs compounds in addition to water, they should differ in this respect. It should be pointed out that Ketchel and Williams attempted unsuccessfully to absorb the "volatile factor" with Ringer's solution, dilute alkali, and acid. They were partially successful with activated charcoal and, possibly, with mineral oil. The major constituent absorbed by silica gel and copper sulfate is water. It appears that the addition of small amounts of active desiccant to the cultures lowered the percentage of cyst differentiation, an effect not produced by water-saturated desiccants.

To summarize, a reinvestigation of the "volatile factor" proposed by Ketchel and Williams (*1*) was carried out, a number of their experiments being repeated under presumably identical conditions. One exception to the complete uniformity was that blood and cysts used here were derived from *Samia cynthia,* a species closely related to *Hyalophora* (*Samia*) *cecropia.* This difference of material could not have affected the results, since Schmidt and Williams (*4*) have reported that *H. cecropia* and *S. cynthia* blood and germinal cysts behave identically in culture to the extent that the cysts of each species can differentiate in the blood of the other. Despite the several similarities of experimental results, our study indicates that the "volatile factor" is

Table 1. Effectiveness of water and other substances in promoting insect spermatocyte differentiation in large culture chambers.

Cultures with active blood and cysts (No.)	Conditions	Drops of other liquid (No.)	Experiments (No.)	Cysts developed at 5 days (%)
	Series 1a. Development of cysts in a single drop of active blood			
1	Sealed depression slide		14	42
	*Series 1b. Development of cysts in active blood in the presence of other active cultures**			
1			6	0
2			4	4
4			4	13
6			2	42
8			3	35
12			2	45
16			2	52
	*Series 1c. Development of cysts in active blood in the presence of water**			
1		0	2	0
1		1	2	7
1		3	7	11
1		5	2	44
1		7	7	56
1		9	2	54
1		11	2	54
1		13	2	40
1		15	7	58
	*Series 2. Development of cysts in active blood in the presence of complete and incomplete cultures**			
1	Active blood and cysts	15	3	42
1	Inactive blood, no cysts	15	4	41
1	Active blood, no cysts	15	3	42
1	Inactive blood and cysts	15	2	46
	Series 3. Differentiation of cysts in active blood in the presence or absence of sufficient quantities of water†			
1	No water supplement		4	0
1	Water supplement (5 ml)		4	45
	Series 4. Development of cysts in the presence of increasing numbers of active cultures, with an excess of water			
1	Depression slide		3	35
1	Water (5 ml)*		4	36
2	Water (5 ml)*		2	33
4	Water (5 ml)*		2	45
8	Water (5 ml)*		1	36
16	Water (16 drops)*		2	35
	*Series 5a. Development of cysts in active blood in the presence of silica gel (0.1 to 0.2 g)**			
4	Dehydrated SiO_2		2	6
8	Dehydrated SiO_2		2	9
4	Hydrated SiO_2		2	20
8	Hydrated SiO_2		2	41
	*Series 5b. Development of cysts in active blood in the presence of copper sulfate (0.1 to 0.2 g)**			
4	Dehydrated $CuSO_4$		2	0
8	Dehydrated $CuSO_4$		2	0
4	Hydrated $CuSO_4$		2	23
8	Hydrated $CuSO_4$		2	37

* In a 60-mm petri dish (volume about 30 cm^3). † In a 100-mm petri dish (volume about 100 cm^3).

water. No other interpretation appears possible.

This interpretation explains not only all of our results but also some results of the earlier workers which heretofore have not been adequately explained. To cite just one example, the observation that the differentiation of cysts in 16 drops of active blood was greatly enhanced above the value obtained for the same number of cysts in two drops can be interpreted as simply a consequence of the larger amount of water available (*5*).

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5. This work was supported in part by the National Science Foundation (grant No. G9833). Dr. Laufer was a fellow of the National Research Council from 1957 to 1959, at the Department of Embryology, Carnegie Institution of Washington, Baltimore, Md.; Mr. Berman's work was supported in part by an undergraduate biological research award (G3383) from the National Science Foundation.

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Punishment in the Squirrel Monkey Saimiri sciurea

Abstract. Punishment has been found not only to suppress the rate of a food-maintained operant response in the squirrel monkey under conditions of high deprivation but to inhibit the emission of that response for 50 days (400 hours) after the punishment has been withdrawn.

Since the publication of Estes's "An Experimental Study of Punishment" (*1*), the effects of presenting a severely aversive stimulus, such as a strong electric shock, contingent upon the emission of a response have been generally regarded as temporary. When shocks were of such an intensity as to completely suppress the rate of bar-pressing in the rat, Estes found that recovery occurred when the punishing stimulus was withdrawn. Recently, Azrin (*2*) found that during prolonged exposure to punishment the rate of a pigeon's key-pecking in the presence of shock returned to a level comparable to that observed before the introduction of punishment. In pigeons subjected to very severe current intensities, however, Azrin (*3*) reported recovery only about 12 days after the shock had been removed.

Little is known about the effects of punishment on the behavior of higher organisms, such as the monkey, although a considerable amount of research on a related phenomenon, conditioned suppression, has been reported, most recently by Brady (*4*) and Sidman (*5*). In this experiment I used a technique similar to that of Azrin, with squirrel monkeys as subjects, in an attempt to extend the earlier findings to a wider variety of species (*6*).

Two experimentally naive, adult, short-haired squirrel monkeys (*Saimiri sciurea*) were starved to 80 percent of the body weights they had had on a free-feeding regimen and were then conditioned to press a lever for food rewards. During the initial stages of training every response was reinforced; later, responding was reinforced intermittently, with mean intervals of first 1, then 3, and finally, 6 minutes between rewards (VI-1, VI-3, and VI-6). After the rate of response had been stabilized on the VI-6 schedule, the experiment was begun. Table 1 summarizes the procedures and results.

Each daily session lasted for 8 hours, so that, although the number of animals available was small, a considerable amount of data were gathered (560 hours for each monkey). On days 1 to 7 the subjects were run on the VI-6 schedule; no punishments were given. During this period an average of 2846 responses were made each day by monkey No. 19 and 2246 by monkey No. 20.

On day 8 the punishment procedure was instituted. After each response, an electric shock of 1-ma intensity and 500-msec duration was given through a grid floor, through the walls of the box in which the monkeys were run, and through the lever. The number of responses in 8 hours was 65 for monkey No. 19 and 29 for monkey No. 20. During the next 11 days the same procedure was in effect, and the number of responses fell to an average of nearly one per day for both animals. This means that the monkeys obtained virtually no food for 8 hours; their weights therefore declined rapidly (as mentioned above, the monkeys were at 80 percent of their normal weights before the experiment began). The weights were allowed to fall an additional 100 g (to 60 percent of normal) before the animals were given food in their home cages, to prevent death and in order to continue the experiment. Punishment was withdrawn on the 20th day, and during succeeding sessions the mean interval between reinforcements was decreased. There was no recovery in the rate of response during the next 50 days (400 hours); then the experiment was terminated.

The monkeys, after punishment was instituted, and thereafter, showed an unwillingness to enter the experimental chamber and what could probably be described as "fearful" behavior when they were finally placed in the box. They would crouch in the corner farthest from the lever and would remain there during the entire session. This behavior persisted throughout the 50 days from the termination of punishment until the end of the experiment.

Table 1. Summary of procedures and results. CRF, every response reinforced.

Schedule of reinforcement	Punishment condition	Mean No. of responses per session	
		Monkey No. 19	Monkey No. 20
	Days 1–7		
VI-6	Off	2846	2246
	Day 8		
VI-6	On	65	29
	Days 9–19		
VI-6	On	1.4	0.9
	Day 20		
VI-6	Off	0	0
	Days 21–34		
VI-1	Off	1.3	0
	Days 35–55		
VI-½	Off	0.8	0.6
	Days 56–70		
CRF	Off	1	0.9

It would appear that the squirrel monkey does not recover from the effects of punishment as do the pigeon and the rat. However, this is not certain, since there were differences other than that of species between the conditions of the study reported here and conditions of the studies of Estes and Azrin—for example, differences in shock intensity and duration, method of presentation of shock, and frequency of food reinforcement. It does, however, seem reasonable to suppose that the monkey may be more sensitive than the pigeon and may therefore be more like man. If this is correct (and further research is needed before any definite conclusions are drawn), severe punishment may have the effect not only of eliminating any desired response but also of permanently inhibiting adaptive behavior in higher organisms.

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