input sometimes produced an entirely different effect on the response (Fig. 1*E*). As soon as a clicking noise was introduced, the amplitude of the response increased significantly, and it remained high throughout the clicking. However, such augmentation or restoration of the response became less and less marked as the novelty of the stimulus gradually decreased through repetition. This type of response modification thus appears to be due to the shift of attention from some previous subject of preoccupation to the novel stimulus given.

If we are to conceive of the flattening of the response by an afferent input as an inhibitory process, the sudden reappearance or restoration of the response by some afferent could be considered a manifestation of "disinhibition." Disinhibition may result from interaction between a previously operating inhibitory process and the novel stimulus. The novel stimulus evokes a shift of attention which in itself produces an inhibitory process. Since this latter inhibition is not observed, the two inhibitory processes must have cancelled each other out. External inhibition in classical Pavlovian conditioning may be a somewhat analogous process, since "unexpected" input preceding the conditioning stimulus inhibits the usual effect (*4*).

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4. I wish to thank Dr. John R. Knott, State University of Iowa, for his critical discussion of this work. This study was aided by federal mental health grant No. 609-5-129 and by the Provincial Mental Health Service of British Columbia.

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Volatile Factor in Culture of Insect Spermatocytes

Abstract. The in vitro differentiation of insect spermatocytes is critically dependent on the water content of the medium. The postulated "volatile factor" is accounted for in terms of a loss of water from the cultures.

When spermatocytes of diapausing pupae of the Cecropia silkworm are cultured in single hanging or sitting drops of the blood of a developing adult, they develop into spermatids and spermatozoa (*1*). It has been reported (*2*) that these striking cytological events take place only if the cultures are enclosed in a tightly sealed chamber. An examination of this phenomenon (*2*) suggested that the developing spermatocytes produce a "volatile factor" which is necessary for the growth and survival of spermatocytes. The nature of this hypothetical factor has been examined in the present investigation.

Table 1. Effect of daily ventilation of culture chambers on development of spermatocytes.

Cultures per chamber	Gas mixture (%)	Cysts counted (No.)	Developing cysts (%)
	*Cyst suspension I**		
4	Air†	804	22 (18–25)
4	O_2(20), N_2(80)‡	708	26 (12–33)
4	O_2(20), N_2(80)	901	26 (15–37)
4	O_2(10), CO_2(5), N_2(85)	606	34 (25–42)
8	Air†	1220	38 (36–46)
8	O_2(20), N_2(80)‡	1287	33 (24–40)
8	O_2(20), N_2(80)	1519	37 (35–41)
8	O_2(10), CO_2(5), N_2(85)	1659	41 (35–48)
16	Air†	1061	36 (28–55)
	Cyst suspension II		
16	Air†	260	53 (46–58)
16	O_2(2), N_2(98)	391	60 (55–62)
16	O_2(2), CO_2(5), N_2(93)	584	58 (53–63)

* Blood diluted with insect Ringer. † Not ventilated. ‡ Not saturated with water vapor.

The cultures were prepared essentially by techniques which have been described (*2, 3*). Each culture chamber consisted of a Lucite ring sandwiched between two glass plates, the joints being sealed with melted paraffin. The internal volume of each chamber was 16 cm^3. In experiments where the chamber was ventilated, the Lucite ring was provided with inlet and outlet tubes. Up to 16 sitting-drop cultures were placed in a grid pattern in each chamber. The drops were of uniform size, but the number of cysts (*4*) in each drop varied between 50 and 300. In each case the experimental and control chambers were prepared from the same suspension of cysts. After 7 days at 25°C, four or more drop cultures were photographed. The degree of development was assessed from the photographs in terms of the percentage of cysts that had completed the meiotic divisions and had begun to elongate.

Table 1 is a summary of the results obtained in cultures ventilated daily with 125 cm^3 of the specific gas mixture given. The mixtures were saturated with water vapor before they were passed into the chamber, except in the two cases noted. The results in Table 1 show no indication of any harmful effects of the ventilation procedure.

One culture was ventilated continuously for 7 days with a gas mixture of 3 percent oxygen, 5 percent carbon dioxide, and 92 percent nitrogen equilibrated with insect Ringer's solution. The flow rate was 30 to 80 cm^3/hr. The average development in two unventilated control chambers was 25 percent, whereas the ventilated chamber showed an average development of 28 percent.

The concept of a "volatile factor" was initially derived from the observation that single drop cultures undergo little or no development if placed in a chamber in the absence of other cultures (*2*). This finding has been confirmed in the present study. Thus, a single culture enclosed in the 16 cm^3 chamber invariably degenerates. If, however, the single culture is placed in a chamber with 7 or 15 drops of blood, it may show development comparable to a drop culture enclosed in a chamber with 7 or 15 other cultures (Table 2).

In this culture technique, water always evaporates from the cultures and condenses as a visible film on the walls of the chamber. The distillation from the drop cultures is attributed to temperature gradients within the chambers, especially when the latter are examined under the compound microscope. I have also been able to show that the blood of the developing adult is ordinarily at the upper limit of concentration that can be tolerated by spermatocytes;

Table 2. Development of single drop cultures accompanied by drops of blood, compared with development of 8 or 16 cultures of the same cyst suspension.

Cultures per chamber	Cysts counted	Developing cysts (%)
8	1393	29 (25–35)
1*	217	32
8	1220	38 (34–46)
1*	201	30
16	402	43 (31–59)
1†	92	66
16	282	30 (12–53)
1†	289	18

* One drop culture plus 7 drops of blood.
† One drop culture plus 15 drops of blood.

therefore, any evaporation may render it toxic to the cysts. These observations and the experiments presented above indicate that it is unnecessary to postulate any "volatile factor" other than water vapor (*5*).

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4. The spermatocytes of the Cecropia silkworm occur as hollow balls of several hundred cells surrounded by follicle cells. Such an aggregation of cells is called a spermatocyst. The cells within one cyst develop synchronously.
5. I am grateful to Prof. C. M. Williams for his very helpful criticism of the manuscript.

8 July 1960

Changes in Incidence of Sex Chromatin in Subcultured Cells

Abstract. Sex chromatin counts of subcultured cells of both female human mammary tumor and female rabbit kidney show a considerable drop from an initial high level. Cultures in which sex chromatin persists also retain the viral insensitivity of their source material.

The presence of sex chromatin in tissue of female origin has been demonstrated in explants and primary trypsinized cell cultures (*1–3*), but generally not in cell lines after prolonged cultivation in vitro (*1, 4*).

The first report of sex chromatin in subcultured female cells was in human mammary tumor tissue cultured for 10 weeks, through nine transfers (*1*). In another report, sex chromatin was not found in human female tissue cultures aged 2½ months to 3½ years, after nine or more transfers, but was present in "younger" cultures aged 2 to 55 days, after three to five transfers (*5*).

More recently, the presence of sex-chromatin-like chromocenters in long-term cultures of both female and male human tissues was reported (*6*). Such chromocenters were found in 5 percent of the HeLa cells studied, and in 27 percent of the D-189 cells, of male origin. Multiple chromocenters, resembling those found in D-189 and, presumably in HeLa, were also found in cell nuclei in a study of 15 successive passages of stock HeLa cells maintained in our laboratory, but we found no typical sex chromatin, as described by Barr *et al.* (*7*).

Since the reported "sex-chromatin-like chromocenters" were found in male as well as in female subcultures, in contrast to other negative findings of sex chromatin in cultures of male origin (*5*), these counts may have included chromatin clumps simulating sex chromatin. The alteration of the distribution of sex chromatin normally found in explants and primary cultures that occurs with prolonged subculturing could be due either to actual loss of sex chromatin or to its masking by increased clumping of the chromatin granules. In either case, these nuclear changes could provide a useful marker of in vitro cell transformation.

The human mammary tumor cell line in which the presence of sex chromatin was first reported (*1*) subsequently was maintained through 24 weeks and 18 transfers, in Lactal (*8*) with human, horse, or rabbit serum. During the subculturing, sex chromatin dropped steadily, from a high of 63 percent to a final count of 9 percent. After the 19th transfer, this culture failed. Therefore it seemed desirable to follow sex chromatin changes in an uninterrupted culture.

Accordingly, counts have been made of the sex chromatin in a line of rabbit kidney cells from their initial cultivation to the present. These cells started as a trypsinized suspension of kidney from a female New Zealand rabbit. The medium was a modified Lactal with cow serum (*9, 10*). At the time of the last count, this culture had surpassed the human mammary tumor line in both duration of maintenance (38th week) and number of transfers (20th). Its continuing vigor is indicated by the fact that at the third day after transfer the number of cells was doubling in 24 hours and an average of 10 percent of the cells were undergoing mitosis.

The following characteristics found in primary explants were used as criteria for sex chromatin in the nuclei of the subcultured cells: (i) one to three well-defined nucleoli in nucleoplasm composed of relatively fine granules which are Feulgen-positive and deeply basophilic with hemotoxylin-eosin staining, and (ii) a wedge-shaped or plano-convex sex chromatin mass, larger than any of the Feulgen-positive granules and more basophilic than the nucleoli. Nuclei with large multiple chromatin clumps were not counted.

Sex chromatin both adjacent to and free of the nuclear membrane was counted in 200 cells from duplicate cultures from each passage, with the exception of the last count (38th week) of the rabbit kidney cells. Because the incidence of sex chromatin was declining, the procedure for that count was modified to reduce the possibility of counting chromatin clumps of any size. Duplicate cultures of two successive days of cultivation were fixed and stained with hemotoxylin and eosin, and in each slide 25 random fields were examined at a magnification of 1000. Only the most typical sex chromatin masses were counted in nuclei with the specified characteristics, and even deeply stained chromatin masses were rejected if they were small, spherical, or not adjacent to the nuclear membrane. Sex chromatin was found in all four of the cultures, and the average did not vary significantly from earlier counts (Fig. 1).

As Fig. 2 shows, there was no significant change, between the primary

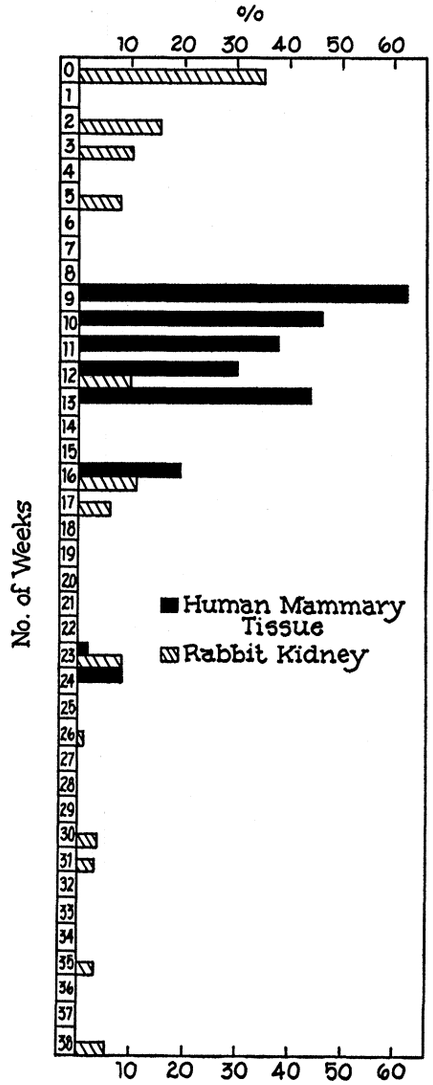

Fig. 1. Percentage of sex chromatin in maintained cultures of female human mammary tumor and of female rabbit kidney cells, by number of weeks. Percentages are average counts of duplicate cultures fixed and stained 3 to 5 days after transfer. Human mammary tumor cultures were not available for counting until the 9th week, representing the 5th transfer. They terminated during the 25th week, after the 19th transfer. Rabbit kidney cultures at the 38th week represent the 20th transfer.