therefore propose that exclusion of calcium from sieve tubes is part of the developmental process whereby they attain the relatively structureless condition which presumably fits them for the function of longitudinal conduction of a flowing solution. The characteristic immobility of calcium in the phloem, with the attendant inefficient distribution of this nutrient in the plant, is on this view the evolutionary price paid for the adaptation of sieve tubes as relatively open, unstructured conduits through which a solution of sugars and other nutrients is free to flow. The one membrane which is fully functional – the plasmalemma – abuts on the cell wall and is therefore exposed to calcium present in the extracellular space or 'apoplast'.

Boron. Like calcium, boron is an essential nutrient; a deficiency of it has prompt and often disastrous effects on growing points and hence, on the entire plant. Its failure to be redistributed via the phloem is apparently disadvantageous. However, I propose that a deficiency of it in sieve tubes, like calcium deficiency, is connected with the role of sieve tubes as conducting elements. The pores connecting sieve tube protoplasts through the intervening walls are often lined with a sleeve of callose which tends to narrow these openings. Recent evidence[1] is to the effect that unless sieve tubes are injured, the sleeves of callose are not sufficiently thick to cause a critical narrowing of the pores. However, various injurious agents including borate[6] cause callose to be deposited more copiously than is otherwise the case, narrowing the pores and in extreme cases, blocking them completely. Like exclusion of calcium, therefore, exclusion of boron from sieve tubes may be an adaptation favoring their maintenance as open conduits.

Part of the above hypothesis (that concerning calcium) has been briefly referred to before[2].

Résumé. Contrairement à la plupart des éléments nutritifs, le calcium et le bore sont en grande partie exclus des tubes criblés et par conséquent ne sont pas efficacement distribués dans la plante, ce qui est apparemment désavantageux. On suppose que l'exclusion de ces deux éléments des tubes criblés favorise leur maintien comme conduits ouverts.

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[6] W. ESCHRICH, H. B. CURRIER, S. YAMAGUCHI, R. B. MCNAIRN, Planta *65*, 49 (1965).

[7] I thank ALDEN S. CRAFTS and H. B. CURRIER for reviewing the manuscript.

PRO EXPERIMENTIS

A Method for Freezing Living Ovaries of *Drosophila melanogaster* Larvae and Its Application to the Storage of Mutant Stocks

The isolation of *Drosophila* mutants has been hindered by the fact that no satisfactory method is available for preserving living flies, so that the stocks have to be maintained constantly.

A method for freezing imaginal disc cultures of *Drosophila* in liquid nitrogen has been reported[1], but it gave variable results when applied to discs taken directly from the larva. Therefore, a new technique for freezing imaginal discs was developed which gives complete survival of the tissue[2]. Encouraged by these results we adapted this method to the freezing of larval ovaries. Transplanted ovaries can establish a connection with the host's oviduct and give rise to offspring[3]. In this preliminary note we report on a method for freezing larval ovaries which can be used for preserving mutant stocks.

The ovaries were dissected from wildtype donor larvae of the late third instar (110 h after egg deposition, at 25 °C) in a drop of balanced saline solution[4], and the adhering fat body was carefully removed by tungsten needles. A dozen ovaries was collected and transferred into a 2 ml ampoule containing 0.5 ml of freezing medium I, which consists of 5% glycerin, 80% Schneider's *Drosophila* medium[5] and 15% bovine serum album in fraction V (2 mg/ml). It proved to be most convenient to transfer the ovaries in a small depression slide[6] (2 mm in diameter, 0.2 mm deep, gold (95%) – nickel (5%) which was dropped directly into the ampoule. This prevents dilution of the medium and loss to the tissue. The ovaries are incubated in freezing medium I for 2 h at room temperature. Afterwards, 0.5 ml of freezing medium II (15% glycerin, 70% Schneider's *Drosophila* medium, 15% bovine serum albumin fraction V [2 mg/ml]) are added, the media are mixed gently, and the ampoule is sealed.

Survival and fertility of frozen ovaries

Experiment	1	2	3	4	5
Number of ovaries frozen	12	12	12	12	12
Number of ovaries re-implanted	11	10	8	11	10
Number of ovaries developing normally	6	6	7	8	3
Number of ovaries reduzed in size	1	2	—	2	4
Number of ovaries with progeny	5	4	4	6	5
Total number of progeny from donor ovaries	27	32	12	31	27
Total number of progeny from host ovaries	110	130	116	161	159

[1] E. GATEFF and H. SCHNEIDERMAN, A. Rep. Lab. Schneiderman, Case Western Reserve University (1968), p. 74.

[2] W. BRÜSCHWEILER, in preparation.

[3] B. EPHRUSSI and G. BEADLE, Bull. biol. Fr. Belg. *69*, 492 (1935).

[4] L. CHAN and W. GEHRING, Proc. Nat. Acad. Sci. USA *68*, 2217 (1971).

[5] Schneider's Drosophila Medium (revised), Grand Island Biological Comp.

[6] Depression slide used for 'freeze-etching', Balzers AG, 9496 Balzers Principality of Liechtenstein.

Heating of the contents of the ampoule has to be avoided. After 30 min of exposure to the combined freezing media at room temperature, the ampoule is inserted into a 'biological freezer'[7] and cooled at a rate of 1 °C/min. 3 h later the ampoule is transferred briefly into a dry ice-acetone bath and there attached to an aluminum holder which has been cooled in advance, in order to avoid warming up of the ampoule. Finally the aluminum holder is transferred into a liquid nitrogen tank for storage.

For thawing, the ampoule holder is put directly into a 37 °C water bath. It is recommended to use a face mask for this purpose, since ampoules which have not been sealed properly can explode. Immediately after thawing, the ampoule is opened and the contents is poured into a 1 ml glass depression slide. Usually, at least 10 of the ovaries are recovered and injected directly into genetically marked (*e mwh*)[8] female host larvae of the middle third instar (80 h after egg deposition, at 25 °C). There is no need to wash the ovaries prior to injection since the freezing medium is not toxic. After metamorphosis the host fly can be examined for the presence of the donor ovary. An additional ovary of normal size is found in 60% of the host females, in 20% the additional ovary is reduced in size, and in another 20% it is absent.

By mating the host females to genetically marked (*e mwh*) males, it can be shown that the donor ovary establishes contact with a host oviduct in slightly fewer than $^2/_3$ of the cases, which would be expected if the donor ovaries were to attach at random. The donor ovary produces on the average only 20% offspring as compared to the host ovary, but the donor offspring is viable and fertile. In the Table the data for a series of typical experiments are listed. The reduced number of offspring is also observed when control ovaries, which have not been frozen, are transplanted, indicating that it is the transplantation rather than the freezing technique which reduces the number of offspring. It may be advantageous to use mutants lacking ovaries as hosts in order to reduce competition between the implanted and the host ovaries.

The production of viable and fertile offspring by the ovary which has been frozen, thawed and transplanted allows us to use this method for storing mutant stocks. How long the ovaries can be stored in liquid nitrogen without damage, remains to be seen. A detailed report of this work and its possible application to the freezing of testis will be published later[2].

Zusammenfassung. Eine Methode zum Gefrieren lebender Ovarien von *Drosophila melanogaster*-Larven wird beschrieben. Diese Gefrierkonservierung ist geeignet, Mutantenstämme aufzubewahren.

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[7] 'Biological Freezer' BF-6, Union Carbide Comp.

[8] D. Lindsley and E. Grell, *Genetic variations of Drosophila melanogaster* (Carnegie Inst. Wash. Publ. No. 627, 1968).

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A Simplified Silver Impregnation Method

The methods of Nauta and Gygax[1] and Fink and Heimer[2] for the silver impregnation of anterograde degeneration have been a great advance in the research of the central nervous system. The technical processes involved in these methods, however, require a lot of time. Besides, we have proved that at times they do not give good results in brains which have been kept in formaldehyde for a long time.

Silver impregnation methods in general give variable results[3]. This is due to the large number of physical and chemical factors involved[4]. However, some data must be taken into consideration: a) According to Jacobson[5], sensitivity to the impregnation increases if potassium permanganate is left out in the pretreatment. b) Auto-oxidation phenomena are produced in tissues which have been kept a long time in formaldehyde, and hence it is innecessary to use potassium permanganate[6,7]. c) The silver impregnation methods can be further simplified by omitting the initial reaction of the tissues with aqueous silver[8].

Bearing in mind these data, and hoping to find a simplified method which can also be used for brains kept for a long time in formaldehyde, we carried out several tests, and arrived at the following method:

1. Distilled water. 2. 2'5% uranyl nitrate, 5 min. 3. Distilled water. 4. Ammoniacal silver: 30 ml of 2.5% silver nitrate, 1–1.5 ml of concentrated ammonium hydroxide, 1.8 ml of 2.5% sodium hydroxide, 15–20 min. 5. Reducer: 800 ml of distilled water, 90 ml of 100% ethyl alcohol, 27 ml of 1% citric acid, 27 ml of 10% formalin, briefly. 6. Reducer: the same reducer, briefly. 7. Distilled water. 8. 1% sodium thiosulfate, 2 min 9. Distilled water.

By using 0.025% potassium permanganate and 1% hydroquinone, either independently or both at the same time, the pretreatment has been shortened. We have observed, in accordance with Nauta and Gygax[1], that suppresion is produced when potassium permanganate is used (Figure 1). When used on its own, hydroquinone-oxalic acid seems to increase both the number of fibres impregnated and the intensity of the impregnation. Since hydroquinone-oxalic acid produces reduction, it is sufficient to use it alone in the pretreatment when the auto-oxidation phenomena are prolonged. In such cases, leaving out permangante and hydroquinone-oxalic acid gives a good result (Figure 2). Otherwise, it is necessary to use either potassium permanganate and hydroquinone-oxalic acid together in adequate proportions, or potassium permanganate alone.

As suggested by Eager[8], we obtained better results by using uranyl nitrate in the pretreatment. It also seems

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