biochemical studies. COUPLAND[12] suggested that electron-dense bodies in rat adrenal medulla (which morphologically resemble those seen in our micrographs) were lysosomes. Our biochemical work and histochemical studies with the electron microscope confirm the interpretation that in the adrenal medulla there are three distinct cell organelles: mitochondria, chromaffin granules and lysosomes.

Zusammenfassung. Die Gomori-Reaktion für saure Phosphatase wurde an Nebennierenmarkschnitten von Rindern durchgeführt. Ihre elektronenmikroskopische Untersuchung ergab die Lokalisierung der Enzymaktivität in den Lysosomen. Chromaffine Granula und Mitochondrien zeigten keine Aktivität.

S. BRADBURY, A. D. SMITH, and H. WINKLER

University Departments of Human Anatomy and Pharmacology, Oxford (England), November 1, 1965.

[12] R. E. COUPLAND, J. Anat., Lond. *99*, 231.

Changes of Chromosome Number in Cells of *Drosophila melanogaster* Cultured in vitro

It is well known from investigations on mammalian chromosomes that the karyotype of diploid cells cultured in vitro tends to become heteroploid as the culture ages (HSU and MOORHEAD[1,2]). The occurrence of heteroploidy may be related to the other changes in the growing cells as they become adapted to the artificial conditions of the culture. Obviously, such variability in chromosome number is a serious limitation to genetical analysis of cultured cells, because stability of the karyotype is a prerequisite for such studies (DE CARLI, MAIO, NUZZO, and BENERECETTI[3]).

Two hypotheses have been proposed to explain the changes of chromosome number that occur in cultured cells (WESTWOOD and TITMUSS[4], RUDDLE, BERMAN, and STULBERG[5]): (a) heteroploidy may result from non-disjunctions and other mitotic abnormalities, or (b) heteroploidy may be a secondary phenomenon, consisting of losses of individual chromosomes following a primary occurrence of polyploidy.

Since it is difficult in mammalian cells to identify all chromosome pairs unambiguously, no definite choice between these alternatives can be made as yet.

Cells of *Drosophila melanogaster* could be favourable material for studying variations in karyotype, since the identification of each of the 4 chromosome pairs is easy. Although attempts to obtain dividing cells of *Drosophila* in vitro have been largely unsuccessful for many years, there is now an excellent technique for culturing embryonic cells which continue to grow and divide at a high rate for long periods of time (HORIKAWA and FOX[6]). The data on variations of chromosome number of cultured cells of this insect, however, are still scanty.

The technique used in this study was that of HORIKAWA and FOX[6]. Eggs of the Varese wild strain laid over a period of 6 h were used for obtaining the embryonic cells. These were placed in H-5 medium supplemented with 10% new-born calf serum, and cultured at 30 °C. The chromosome analyses were made on squashes prepared 12, 18, 21, 24, 48, 72, 96 and 120 h after the cultures were begun. The cells from each culture were pretreated in an hypotonic solution of 1% sodium citrate for 10 min, then stained in aceto-lactic orcein (OSTER and BALABAN[7]) for 25–30 min. Chromosome counts were made on metaphases; only those which could be drawn unambiguously have been considered.

One to four cultures were made for each time interval, and because of the homogeneity of results from replicates the data obtained have been pooled (Table). In any case the number of cells analysed is more important than the number of cultures, since each culture contains cells derived from 2000–3000 eggs. In the first analysis (12 h), some heteroploid cells (11.9%) are already present in the culture. The frequency of heteroploid cells increases to 29.7% at 18 h, but no tetraploid cells were found. This value remains nearly constant for 48 h. During the interval between 48 and 72 h, the percentage of heteroploidy increases to 78.5% and then remains practically stable until 120 h (Figure 1). Polyploid cells were virtually ab-

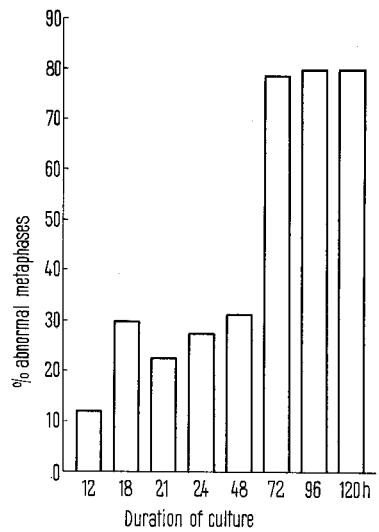

Fig. 1. Changes in percentage of abnormal metaphases of embryonic cultured cells at different stages of culture.

[1] T. C. HSU and P. S. MOORHEAD, Ann. N.Y. Acad. Sci. *63*, 1083 (1956).
[2] T. C. HSU and P. S. MOORHEAD, J. Nat. Cancer Inst. *18*, 463 (1957).
[3] L. DE CARLI, J. J. MAIO, F. NUZZO, and A. S. BENERECETTI, Cold Spring Harbor Symp. Quant. Biol. *29*, 223 (1964).
[4] J. C. N. WESTWOOD and D. H. J. TITMUSS, Brit. J. exp. Path. *38*, 587 (1957).
[5] F. H. RUDDLE, L. BERMAN, and C. S. STULBERG, Cancer Res. *18*, 1048 (1958).
[6] M. HORIKAWA and A. S. FOX, Science *145*, 1437 (1964).
[7] I. I. OSTER and G. BALABAN, Drosophila Information Service *37*, 142 (1963).

Percentage of metaphases showing various chromosome numbers after in vitro culture of *Drosophila melanogaster* embryonic cells

Duration of culture in h	Number of chromosomes													No. of metaphases analysed
	4	5	6	7	8	9	10	11	12	13	14	15	16	
12	–	–	–	3.0	88.1	5.2	3.0	0.7	–	–	–	–	–	135
18	1.6	–	1.6	6.3	71.9	7.8	4.7	–	–	1.6	–	4.7	–	64
21	–	–	1.7	10.3	79.3	1.7	3.4	–	–	1.7	–	–	1.7	58
24	–	1.3	2.5	7.5	72.5	10.0	3.8	1.3	1.3	–	–	–	–	80
48	–	1.6	1.6	8.2	70.5	6.6	3.3	6.6	1.6	–	–	–	–	61
72	–	12.3	4.6	29.2	24.6	15.4	7.7	–	–	3.1	3.1	–	–	65
96	–	5.0	11.7	26.7	20.0	15.0	6.7	5.0	6.7	–	–	1.7	1.7	60
120	–	10.0	3.3	38.3	20.0	20.0	–	–	1.7	3.3	3.3	–	–	60

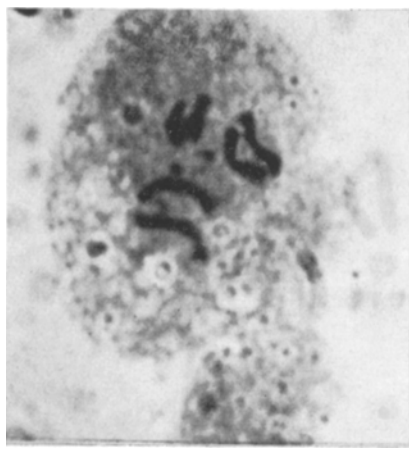

Fig. 2. *D. melanogaster* embryonic cultured cells, stained with aceto-lactic orcein. Normal metaphase after 24 h culture.

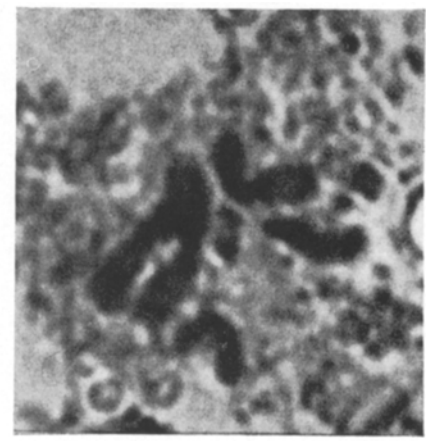

Fig. 4. *D. melanogaster* embryonic cultured cells stained with aceto-lactic orcein. Abnormal metaphase with only one element (X) of the first pair (12 h culture).

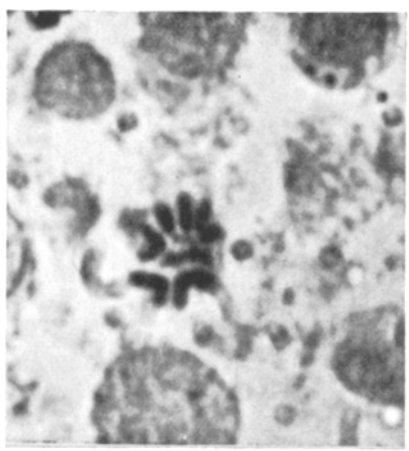

Fig. 3. *D. melanogaster* embryonic cultured cells stained with aceto-lactic orcein. Abnormal metaphase with three elements of the first pair (24 h culture).

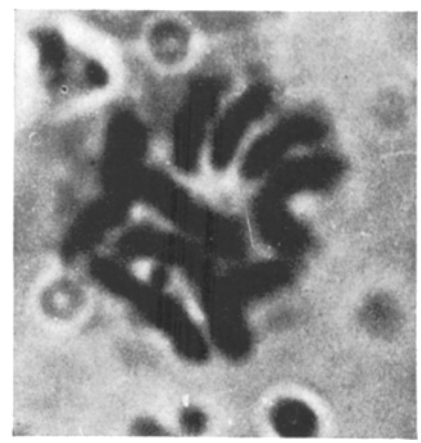

Fig. 5. *D. melanogaster* embryonic cultured cells stained with aceto-lactic orcein. Abnormal metaphase with five elements of the first pair (48 h culture).

sent in all stages. Hence there are two periods of striking increase in heteroploidy, the first between 12 and 18 h and the second between 48 and 72 h of culture.

The main features of these results may be interpreted in the following way: (1) The abnormal metaphases found at 12 h may indicate that some abnormalities occur under normal conditions, and that possibly cells with abnormal chromosome numbers divide more often in vitro than in situ. (2) The increase in frequency of heteroploid karyotypes may be the result of the artificial conditions, which favour heteroploid cells. (3) The rapid increase in heteroploid karyotypes between 48 and 72 h

may be caused by sudden changes in the culture medium, involving nutritional deficiencies. After this time (from 72 to 120 h) chromosomes appear much contracted. As we have already pointed out, the abnormalities are exclusively numerical; structural changes have been found, but are not considered here. The first and fourth pairs are involved primarily when supernumeraries are present or when chromosomes are lost (84.4%), while in only 15.6% of the cells are the two major autosomes involved (Figures 2–5). This finding is worth noting in view of further biochemical studies on cells in vitro. In fact, a different gene dosage is expected for genes located in the second and in the third pair, if compared with those located in the first and the fourth pair.

We may conclude that in *Drosophila* heteroploidy commonly occurs in cultured cells, but that this condition does not follow a primary acquisition of tetraploidy. Nondisjunctions and errors of chromosome distribution are presumably the causes of the numerical abnormalities described. The present data, owing to the scope of this investigation, do not allow any comparison with those found by HORIKAWA and FOX[6]. We wish only to emphasize that these authors distinguish two types of cells (the large and the small ones). Our observations, since they refer to short-term cultures, have dealt mainly with large cells; only between 72 and 120 h may we have found also mitoses of small cells. Using our staining technique, however, the problem of distinguishing the two cell types remains unsolved[8,9].

Riassunto. Cellule embrionali di *D. melanogaster*, coltivate in vitro fino a 120 h, presentano un progressivo aumento delle variazioni dei numeri cromosomici interessanti principalmente il I e il IV paio di cromosomi.

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Istituto di Genetica, Università di Milano (Italy), October 4, 1965.

[8] One of us (S.D.) wishes to express her deepest gratitude to Prof. A. S. FOX and to Dr. M. HORIKAWA for the kind hospitality and the valuable help offered to her during her stay at the Department of Genetics, University of Wisconsin, Madison, during the fall of 1964. The authors are also grateful to Prof. A. S. FOX for critical reading of the manuscript. – The financial help of the Consiglio Nazionale delle Ricerche, Roma, is also gratefully acknowledged.

[9] Added to the proofs. During the last months two additional evidences have been found, which substantiate the viability of the cultured cells: growth curves (till 192 h) and incorporation of tritiated thymidine (the latter finding in collaboration with the Euratom Unit for Human Radiation and Cytogenetics, Pavia, directed by Prof. M. FRACCARO).

About a Possible Mechanism Involved in the Shedding of Sea-Urchins

In spite of extensive investigations concerning the stimulus which causes the spawning of ripe sea-urchins[1–7], the problem of shedding in natural conditions is far from being understood as yet. In the experiments described, we therefore tried to elucidate the influence of spawning sea-urchins on other members of a sea-urchin population in their natural environment.

A colony of *Paracentrotus lividus* (Lam.) inhabiting rocky bottom at a depth of 2–3 m was chosen for the experiments in situ. The average density of population was estimated to be about 5 sea-urchins per m^2. This experimental area was in the North Adriatic near Figarola Island. Underwater observations and the removal of sea-urchins during the experiments were accomplished by free diving technique.

Dense suspensions (about 10%) of homogenized ripe male and female gonads from *Paracentrotus lividus* were prepared. The suspension was taken in a syringe of 200 ml. By diving, 5–10 ml of this suspension was delivered from the syringe close to the sea-urchins. The suspension was never delivered nearer than 10 cm to the animals. The effects of the injection of the gonad suspension were inspected by diving. The shedding which occurred in some animals could easily be registered. From the colour of extruded gametes it was easy to conclude whether the reacting animal was a male or a female. No animal was tested twice. During the whole procedure described previously the animals were neither touched nor disturbed in any other way. About 5 min after the gonad suspension was injected, the animals were lifted from the bottom without regard as to whether they reacted to the injection with shedding or not. They were opened with a circular cut and their sex and maturity were established.

In these experiments, more than a hundred animals were tested: 51 males and 50 females. The results of these investigations are summarized in the Table.

The shedding reaction in sea-urchins (*Paracentrotus lividus* Lam.) tested with the suspension of homogenized male and female gonads, respectively

Suspension applied	Reaction	Sex and maturity of tested animals			
		Male		Female	
		Ripe	Unripe	Ripe	Unripe
Male	Shedding	–	–	24	–
	No shedding	22	2	1	4
Female	Shedding	23	–	3	–
	No shedding	4	–	14	4

[1] L. PALMER, Physiol. Zoöl. *10*, 352 (1937).
[2] H. M. FOX, Proc. Camb. phil. Soc. biol. Sci. *1*, 71 (1924).
[3] H. M. FOX, Nature *109*, 237 (1922).
[4] M. YOSHIDA, Annotnes zool. jap. *25*, 117 (1952).
[5] T. MORTENSEN, III. K. danske Vidensk. Selsk. Skr., Naturv. Math. Ser. 9, Vol. 7, 1 (1937).
[6] E. B. HARVEY, *The American Arbacia* (Princeton University Press 1956).
[7] A. MONROY, cited as personal communication in HARVEY's *The American Arbacia*.