PRO EXPERIMENTIS

An Improved Technique of Preparing Primary Cultures of Isolated Cells from Adult Frog Kidney

Attempts to culture amphibian tissues have been made previously by others, but all have employed the hanging drop plasma or lymph clot system. This method is not very suitable for quantitative studies in cytochemistry, due to the nature of growth in the persisting clot. Monolayer cultures of isolated cells can provide a better system for such studies. Recently a method for culturing isolated cells from various cold blooded vertebrates was described by WOLF et al.[1]. Their method primarily designed for fish tissue cultures seemed to work on frog cells to some degree but failed to give us satisfactory growth. Our studies of growth of several hundred explants of certain tissues of salamander and frog in various media have led us to the following method of preparing cultures of isolated cells from frog kidney.

Young, healthy frogs (30–50 g in weight), *Rana pipiens*, were washed in dilute detergent and bathed in 70% ethanol. They were killed by pithing and kidneys were immediately removed asceptically in a sterilized Ringer's saline containing 1000 units Penicillin, 100 µg streptomycin and 100 units mycostatin per ml. The tissue was cut into small pieces (2^3–3^3 mm) and transferred into a trypsinising flask (Bellco[2]) containing 5 ml of a dissociat-

Tab. I. Composition of the dissociating medium

A	B	C	D	E
NaCl 6.8 g KCl 0.1 g Na_2HPO_4 0.110 g	$NaHCO_3$ 0.2 g	Serum Globulin (Bios)[2] 1.0 g	Trypsin 1:250 (Difco)[2] ... 2.5 g	Dextrose ... 1.0 g
500 ml H_2O	100 ml H_2O	100 ml H_2O Bring it to boil 2 to 3 times to dissolve	250 ml H_2O Dissolved, left overnight and filtered	50 ml H_2O

All units dissolved separately, mixed and filtered through millipore filter of 0.45 µ pore size by vacuum. Penicillin G 100000 units; streptomycin sulphate 100 mg; mycostatin 50000 units; and 1 ml solution of 0.5% phenol red added to 1000 ml of the above medium.

Tab. II. Composition of growth medium

Eagle's basal medium modified by replacing the salts with following amounts/l

	g		g
A. NaCl	5.150	C. $NaHCO_3$	0.750
KCl	0.075	D. Lactalbumin hydrolysate (Nutritional Biochem.)[2]	5.000
$MgSO_4 \cdot 7\ H_2O$	0.204		
$Ca(NO_3)_2 \cdot 4\ H_2O$	0.078		
$CaCl_2$	0.045	E. Yeast extract (Nutritional Biochem.)	1.000
B. Na_2HPO_4	0.0300		
KH_2PO_4	0.0375		

Growth medium was made as follows
82% above medium
5% whole egg ultrafiltrate (microbiological associates)[2]
13% calf serum

pH 7.4 adjusted by one or two drops of 1.N. NaOH or 5% CO_2 in both. Antibiotics concentration as shown in Table I.

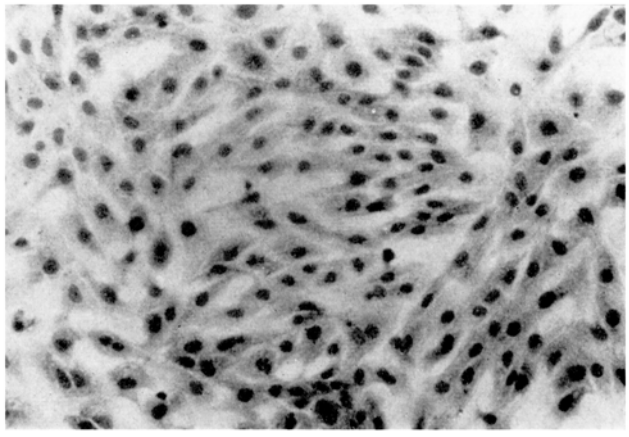

Fig. 1. The cells of kidney culture 7 days old stained by May-Grünwald-Giemsa technique. 125×.

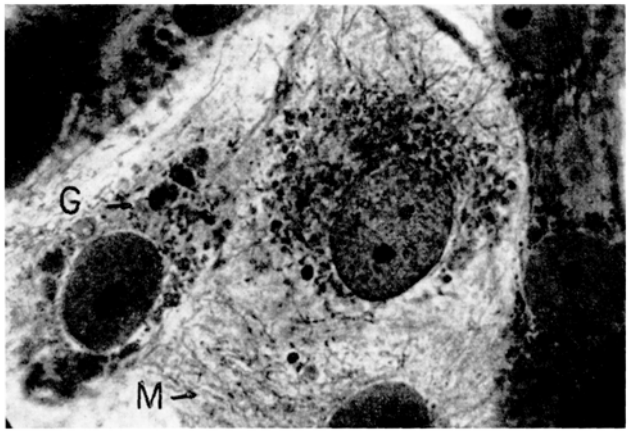

Fig. 2. Mitochondria (M) and Golgi (G) apparatus in cultured cells of frog kidney, fixed by Champy fixative and stained by Kull technique. 1330×.

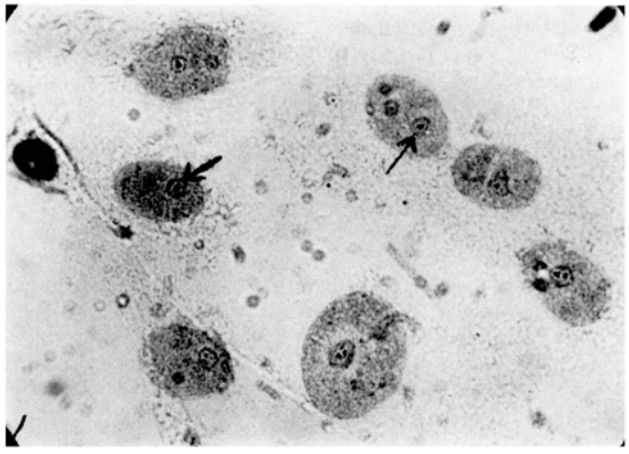

Fig. 3. The cells of frog kidney with Lucke's adenocarcinoma. The nucleoli are found to contain seemingly Feulgen positive granules (arrow). Culture 10 days old. Stained with Feulgen method. 650×.

[1] K. WOLF, M. C. QUIMBY, E. A. PYLE, and R. P. DEXTER, Science *132*, 1890 (1960).

[2] J. PAUL, *Cell and Tissue Culture*, Textbook, 2nd Edition (E. and S. Livingstone, Edinburgh 1960).

ing medium (Table I) per pair of kidneys. This medium was found more satisfactory than the phosphate buffer used by WOLF et al., since it provides faster dissociation and retains more viable cells. The digestion was done over a magnetic stirrer (Bellco) at 12–15°C, maintained by a cold running water bath. The medium was changed twice in the first 2 h and replaced by fresh medium to drain any toxic material. When most tissue was dissociated, the cells were centrifuged at 1000 rpm for 4 min, washed in the Ringer's saline, centrifuged and dispersed in a growth medium (Table II) to give concentration of 3×10^5 cells per ml. They were allowed to attach in 4 oz. medicine bottles or Leighton tubes with 10×50 mm No. 1 coverglasses at 18°C. The above growth medium was used because we had found that the modified Eagle's basal medium, containing salt concentrations used by BARTH[3] for frog embryonic cells, with calf or horse plasma, gave better growth of the amphibian tissue explants than the undiluted or arbitrarily diluted mammalian media or other media.

Most of the viable cells attached within 70 h. In seven to ten days mitotically active (approx. 2.5% mitotic index) cultures were obtained. The cells form a uniform monolayer of predominantly epithelioid cells (Figure 1) containing long threadlike mitochondria (M Figure 2) and one or two juxta nuclear Golgi bodies (G Figure 2).

Frog kidney cells with Lucke's adenocarcinoma (Figure 3) have also been cultured with the above technique and have found to attach and grow faster than the normal cells.

The primary cultures have been kept as long as three months without any noticeable impairment of growth.

Zusammenfassung. Eine verbesserte Technik zur Gewinnung der ersten Einzelschichtbildungen von Froschnierenzellen wurde entwickelt. Die Zellen werden in neuem auflösendem Nährboden isoliert und wachsen auf modifiziertem Eagle-Nährboden mit 13% Kalbsserum. Nach 10 Tagen zeigt sich stark aktives mitotisches Zellwachstum in der Kultur.

V. C. SHAH

Zoology Department, Columbia University, New York (U.S.A.), November 22, 1961.

[3] L. G. BARTH and L. J. BARTH, J. Embryol. Morph. *7*, 210 (1959).

A Quantitative Micro Tube Gel Precipitin Technique

The gel-precipitin technique developed by OUDIN[1] was elaborated by OUCHTERLONY[2] on agar plates. OAKELY and FULTHORPE[3] adapted OUCHTERLONY's modifications to test tubes. Several variations of these techniques have been published[4,5]. A micro adaptation[6] of OUCHTERLONY's plate technique proved superior to conventional methods. Since the test tube technique of OAKELY and FULTHORPE is better adapted for quantitative work[4], and has been found to yield a definable pattern, where many plate modifications failed[7], and since a micro adaptation of this technique has apparently not been attempted, the following technique was devised.

The precipitin pattern is developed in a capillary tube (50 mm × 1, or 2 mm), charged as indicated in Figure 1. First, 1% melted agar is injected into the middle of the capillary with a syringe, up to the required length, and is left to set. The antigen and antibody solutions are then injected at opposite ends of the agar column, carefully avoiding air bubbles and injury to the agar surfaces. Quantities of each reactant solution, as little as 0.02 ml or less, are sufficient. Finally, both ends of the capillary are sealed with melted wax, leaving an air bubble between the wax plug and the reactant solutions.

The development of the precipitin pattern can be conveniently followed by photographic recording, or by tracing after projection through a microfilm reader. After sufficient development of the pattern, the column can be extruded unharmed, and washed into a bath of distilled water. After washing, the agar column can be repacked in a bigger capillary filled with distilled water, sealed with wax, and thus preserved undeformed permanently. Agar columns after washing can be stained, to intensify the pattern[6], or to study specific components[8].

Accurate determination of the relative positions[5,9] and intensities[10] of the various bands are required to verify and apply the theories concerning the formation of the precipitin patterns. In the technique reported here, the positions of all bands can be determined either by a travelling microscope, or an ordinary microscope fitted with a graduated stage, or simply, but with the same degree of accuracy, from measurements on a tracing of the projected pattern. The relative intensities of the various bands were evaluated by scanning the 35 mm negative of the precipitin pattern, using a paper electrophoresis scanner (Spinco Analytrol unit, Beckman Instruments, Inc., Belmont, California).

Figure 2 illustrates the degree of resolution attainable for the system tetanus toxin and tetanus anti-toxin. Successful patterns were obtained with the following antigens against their respective antisera from horses or rabbits: Diphtheria toxin, human serum, cow and other animal sera, milks of different cattle, vaccinia and influenza viruses, and also different snake venoms. This technique may well be applied in the study of other precipitin systems.

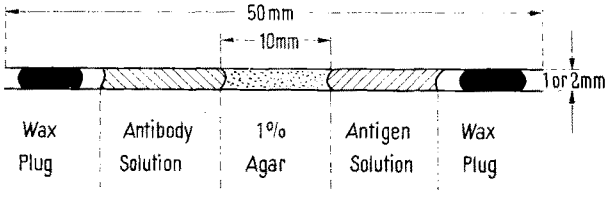

Fig. 1. The capillary precipitin set-up

[1] J. OUDIN, Ann. Inst. Past. *75*, 30 (1948).
[2] Ö. OUCHTERLONY, Acta path. microbiol. scand. *26*, 507 (1949).
[3] C. L. OAKELY and A. J. FULTHORPE, J. Path. Bact. *65*, 49 (1953).
[4] C. L. OAKELY, Discussions, Faraday Soc. *18*, 358 (1954).
[5] A. POLSON, Science Tools *5*, 17 (1958).
[6] A. J. CROWLE, J. lab. clin. Med. *52*, 784 (1958).
[7] W. A. PIERCE, Jr., J. Bact. *77*, 726 (1959).
[8] L. KORNGOLD and R. LIPARI, Science *121*, 170 (1955).
[9] E. L. BECKER and J. C. NEFF, J. Immunol. *83*, 571 (1959).
[10] A. HAYDEN and E. L. BECKER, J. Immunol. *85*, 591 (1959).