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Masahiro Miyazaki* Akiharu Watanabe† Masayuki Shiraishi‡
Teruki Hoshika** Keiko Miyano†† Jiro Sato‡‡

*Okayama University,
†Okayama University,
‡Okayama University,
**Okayama University,
††Okayama University,
‡‡Okayama University,

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Abstract

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KEYWORDS: primary culture, adult rat hepatic cells, hormones, albumin, tyrosine aminotransferase

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PRIMARY CULTURE OF ADULT RAT LIVER CELLS. III. HORMONAL EFFECTS ON CYTOLOGICAL AND BIOCHEMICAL PROPERTIES OF PRIMARY CULTURED CELLS

Masahiro MIYAZAKI, Akiharu WATANABE*, Masayuki SHIRAISHI, Teruki HOSHIKA, Keiko MIYANO and Jiro SATO

*Division of Pathology, Cancer Institute (Director: Prof. J. Sato), and *First Department of Internal medicine (Director: Prof. H. Nagashima), Okayama University Medical School, Okayama 700, Japan*

Abstract. The effect of various hormones on cytological and biochemical properties of cultured hepatic cells were investigated in order to obtain long-term survival of the hepatocytes with adult liver functions in primary culture. Insulin supplementation of the culture medium enhanced the attachment efficiency of cells in primary culture without affecting either maintenance of morphological characters of epithelial cells or retention of liver-specific functions in cultured cells. A combination of dexamethasone and insulin was apparently effective in stimulating the formation of a monolayer of polygonal cells with granular cytoplasm and in maintenance of liver-specific functions for relatively longer periods. Supplementation with either dexamethasone or hydrocortisone alone enhanced tyrosine aminotransferase activities in cultured cells for at least 4 days postinoculation. These steroid hormones also allowed growth of small epithelial cells with clear cytoplasm and maintenance of increased albumin production for 8 days after inoculation. The roles of these hormones in the primary culture of isolated hepatic cells are discussed in the present paper.

Key words: primary culture, adult rat hepatic cells, hormones, albumin, tyrosine aminotransferase

High recovery of viable hepatocytes from adult rat livers is now possible using the recirculating liver-perfusion technique and proteolytic enzymes (1, 2). A primary cell culture with these cells has proven useful in studies on hepatic metabolism and hormonal regulation of protein biosynthesis (3). However, the application of this technique is limited by the fact that functional changes of cells readily occur at an early stage of primary culture (4). A variety of biochemical activities characteristic of normal adult hepatocytes *in vivo* have been reported; *i. e.*, the inducibility of tyrosine aminotransferase (5) and biosynthesis of glycogen (3) and albumin (3, 4). However, the inducibility of tyrosine aminotransferase has been shown to decrease after several days in culture (5). It is still unclear whether this finding results from an inadequacy of culture environments

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or the nature of cultured hepatocytes. Systematic studies on the fate of isolated hepatic cells in culture are obviously needed in order to explain the phenomenon described above.

In a previous report (4), the effects of changing cell inoculum size and adding various sera to the culture medium on the cytological and biochemical properties of primary cultured cells were examined. It was concluded that the maintenance of hepatocytes with adult liver functions in culture could not be achieved by increasing the inoculum cell number alone to inhibit cell proliferation at the early stages of primary culture. Various hormones were added to improve culture conditions and thereby obtain prolonged survival of the hepatocytes with specialized functions in the primary culture. In the present study, effects of these hormones on the cytochemical properties of primary cultured hepatic cells are described with respect to the period of time in culture.

MATERIALS AND METHODS

Animals and chemicals. Three-month-old Donryu male rats, weighing 210 to 290 g, were used in this experiment, which were fed *ad libitum* on Oriental Laboratory Chow MF and water.

Trypsin (1 : 250) was purchased from Difco. Dexamethasone, hydrocortisone-21-sodium succinate (hydrocortisone), insulin and glucagon were obtained from Sigma Chemical Co., and dexamethasone disodium phosphate (dexamethasone) from Nippon Merck Banyu Co.. Steroid hormones were dissolved in water, sterilized by passing through a millipore filter and added to the control culture medium at final concentrations of 2.5×10^{-6} M and 3.0×10^{-6} M, respectively. Insulin and glucagon were dissolved each in a solution containing 0.006 N HCl and 0.01% bovine serum albumin (Fraction V, Sigma Chemical Co.) (6) and similarly sterilized and added to the medium at a final concentration of 1.6×10^{-6} M.

Preparation of isolated hepatic cells. As reported previously (2, 4), isolated hepatic cells were prepared by perfusing rat livers with 0.1% trypsin in Ca^{2+}-free Hanks' balanced salt solution (CF-HBSS) (7) at 37°C for 30 min. Freshly isolated hepatic cells were washed twice with CF-HBSS. The cell viabilities were judged by exclusion of a vital stain, trypan blue (8). The viabilities of the cells used in this experiment were from 60 to 80%.

Primary culture of isolated hepatic cells. Before inoculation, hepatic cells were washed once with the control culture medium, *i. e.*, Eagle's minimal essential medium (MEM, Chiba Pref. Serum Inst.) (9), which was supplemented with 20% bovine serum (BS) inactivated at 56°C for 30 min. Penicillin and streptomycin sulfate were added to the culture medium at final concentrations of 100 units and 100 μg per ml, respectively. The culture medium containing antibiotics was used only for the initial 2 days after inoculation. The hepatic cells were inoculated at a concentration of 35×10^4 cells per ml into 100 mm Falcon plastic dishes (11.1 ml in volume), unless otherwise indicated, and cultured in a

humidified atmosphere of 5% CO_2 and 95% air at 37°C. The spent media were renewed everyday and stored at −20°C for the measurement of albumin contents by a radioimmunoassay using the double antibody method (4). Morphological observation of cultured cells was performed under a phase contrast microscope for 8 days after inoculation.

Measurement of enzyme activities in cultured cells. Activities of glucose 6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9, G6Pase) (10, 11) and tyrosine aminotransferase (L-tyrosine: 2-oxoglutarate aminotransferase, EC 2.1.6.5, TAT) (12) were determined as previously reported (2, 4). Specific activities were expressed in milliunits (nmoles of product formed per min) per mg protein of supernatants for TAT and homogenates for G6Pase. Protein contents were measured by the method of Lowry *et al.* (13).

RESULTS

Morphological characters of cultured hepatic cells. Cytological properties of isolated hepatic cells were studied under different primary culture conditions (photos 1 to 4). Insulin supplementation consistently improved the cell attachment efficiency (photo 1-e) (14). The maximal attachment of hepatic cells to the substratum was observed when the primary culture of isolated hepatic cells were initiated in the presence of dexamethasone and insulin (photo 1-b). These attached cells with granular cytoplasm tightly clustered, having an epithelial appearance (photo 1-b, 2-b and 3-b). Combined supplementation of dexamethasone and insulin was also more effective for survival of the polygonal-granular cells than either one alone (photo 3-b, 3-c and 3-e). When isolated hepatic cells were cultured in the control medium supplemented with either dexamethasone or hydrocortisone, the small epithelial cells with clear cytoplasm (epithelial clear cells) grew selectively and the resulted cultures showed a homogeneous population (photo 4-c and 4-d). Combined supplementation of dexamethasone and insulin delayed the appearance of the clear cells in the primary culture. Morphological findings of hepatic cells cultured in the presence of either insulin or glucagon alone could not be distinguished from those in the control culture at 8th day of culture, those cultures consisting of various shaped cells (Photo 4-a, 4-e and 4-f). Morphological features of cultured hepatic cells under the various conditions at 8th day of culture are summarized in Table 1.

Biochemical properties of cultured hepatic cells. Activities of G6Pase and TAT in cultured hepatic cells under various conditions are illustrated as a function of time in culture (Figs. 1 and 2). Hepatic cells cultured in the presence of either insulin or glucagon exhibited slightly higher activies of G6Pase than those in the absence of pancreatic hormones, 1 day after inoculation. G6Pase activities in cultured cells, however, could not be maintained and decreased rapidly even in the presence of those hormones. Neither dexamethasone nor hydrocortisone had

Table 1. Effect of various hormones on morphological properties of hepatic cells *in vitro*

Hormone added	Final concentration in the medium (M)	Morphology
None	–	Mixed
Dexamethasone	2.5×10^{-6}	Epithelial
Hydrocortisone	3.0×10^{-6}	Epithelial
Insulin	1.6×10^{-6}	Mixed
Glucagon	1.6×10^{-6}	Mixed
Dexamethasone[a] +Insulin	1.0×10^{-5} 1.6×10^{-6}	Epitheloid

Morphological features of cultred cells were judged from the observations of 3 different cultures under a phase contrast microscope.
a Dexamethasone disodium phosphate (dexamethasone).

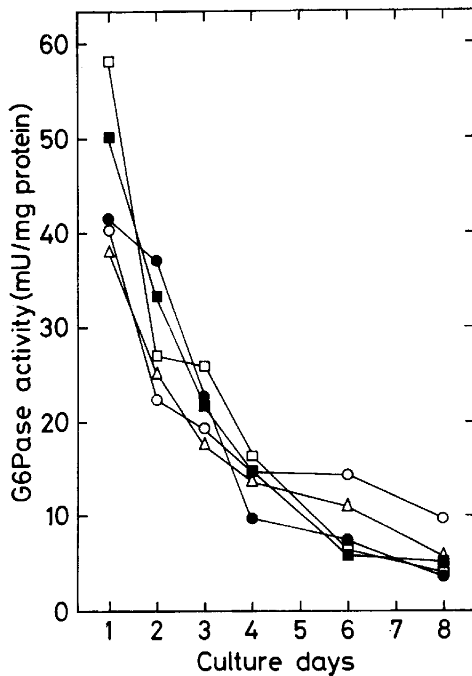

Fig. 1

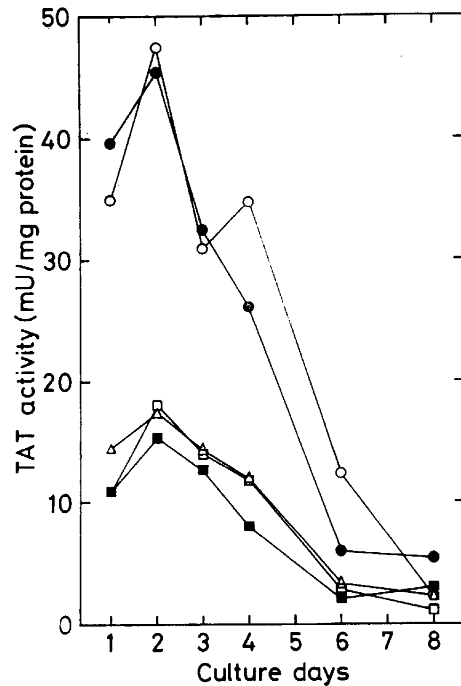

Fig. 2

Fig. 1. Time courses of G6Pase activities in hepatic cells cultured in the media containing various hormones. Cultured cells were pooled from 3 different dishes and homogenized for measuring G6Pase activities. The final concentrations of hormones added are as follows: △–△, control; ○–○, dexamethasone (2.5×10^{-6} M); ●–●, hydrocortisone (3.0×10^{-6} M); □–□, insulin (1.6×10^{-6} M) and ■–■, glucagon (1.6×10^{-6} M).

Fig. 2. Time courses of TAT activities in hepatic cells cultured in the media containing various hormones. Cultured cells were pooled from 3 different dishes and homogenized for determining TAT activities. The final concentrations of hormones added are as follows: △–△, control; ○–○, dexamethasone (2.5×10^{-6} M); ●–●, hydrocortisone (3.0×10^{-6}M); □–□, insulin (1.6×10^{-6} M) and ■–■, glucagon (1.6×10^{-6} M).

any effect on maintenance of G6Pase activity in cultured hepatic cells. TAT activities in hepatic cells, which were cultured in the control medium containing either dexamethasone or hydrocortisone, were much higher than those under any other conditions of culture. TAT activities in the presence of these steroid hormones reached the maximal level 2 days after inoculation. The increased activities were maintained up to 4 days after initiating culture, followed by declining rapidly. The higher activities are probably due to known inductive effect of steroid hormones on this enzyme (5). Neither insulin nor glucagon supplementation exhibited any effects on the activity of TAT in cultured cells, although these peptide hormones induced rat liver TAT *in vivo* (15).

The time courses of albumin production in cultured hepatic cells in media containing various hormones are shown in Table 2. Both dexamethasone and

TABLE 2. EFFECT OF VARIOUS HORMONES ON ALBUMIN CONCENTRATIONS IN THE SPENT MEDIA FROM THE PRIMARY CULTURE OF ADULT RAT HEPATIC CELLS

Incubation time (h)	Albumin concentration (ng/ml)				
	Control	Dexamethasone	Hydrocortisone	Insulin	Glucagon
0–24	3278±380	2636±259	4410±115	6126±112	2800±331
24–48	1041±206	766±89	1193±109	1453±77	510±26
48–72	336±129	443±38	705±70	460±18	133±28
72–96	176±108	468±27	593±90	181±6	60±19
96–120	89±56	543±151	493±67	117±7	53±10
120–144	49±46	523±223	228±62	78±5	37±10
144–168	13±9	261±129	236±116	27±4	21±4
168–192	3±3	240±78	230±126	7±2	11±5

The final concentrations of hormones added are as follows: dexamethasone, 2.5×10^{-6} M; hydrocortisone, 3.0×10^{-6} M; insulin, 1.6×10^{-6} M and glucagon, 1.6×10^{-6} M. Results are expressed as mean ± standard deviation from 3 expreiments.

hydrocortisone markedly increased the production of albumin in cultured hepatic cells. The enhanced production of albumin in these cells was maintained up to 8 days postinoculation. However, neither insulin nor glucagon supplementation had this effect. The simultaneous addition of dexamethasone and insulin resulted in maximally enhanced production of albumin in primary cultured cells (Table 3). This finding seemed to be related to the prolonged survival of the polygonal cells with granular cytoplasm, which could be observed under the combined supplementation of dexamethasone and insulin.

TABLE 3. COMBINED EFFECT OF DEXAMETHASONE AND INSULIN ON ALBUMIN CONTENTS IN THE SPENT MEDIA FROM THE PRIMARY CULTURE OF ADULT RAT HEPATIC CELLS

Incubation time (h)	Albumin concentration (ng/ml)	
	None	Hormones supplemented
0–48	3370±103	14506±854
48–96	716±62	5440±65
96–144	119±5	1088±37
144–192	28±7	416±23

Isolated hepatic cells were inoculated at a concentration of 35×10^4 cells per ml into 60 mm Falcon plastic dishes (4 ml in volume) and cultured. The culture medium was renewed every 2 days. The culture medium was supplemented with or without dexamethasone disodium phosphate and insulin at final concentrations of 1.0×10^{-5} and 1.6×10^{-6} M, respectively. Results are expressed as mean ± standard deviation from 3 experiments.

DISCUSSION

There have been several attempts to prolong the survival of hepatocytes with adult liver functions *in vitro* by investigating the culture conditions (3, 4, 14, 16, 17). In the present experiment, isolated hepatic cells were cultured in the hormone-supplemented media in order to maintain their functional and morphological properties similar to those of the hepatocytes *in vivo* for a longer period. TAT and albumin would be ideal markers to demonstrate that the hepatocytes with specialized liver functions are maintained in culture, because these biochemical parameters are relatively stable in the primary cultures of isolated hepatic cells (4).

It has been reported by Gerschenson *et al.* (6) that the established cell line (RLC), derived from adult rat liver, can be adapted to grow in the serum-free medium when insulin is added to the medium. Insulin supplementation to the control medium enhanced the attachment of isolated hepatic cells in the primary culture, although it had no effect on the survival of hepatocytes with differentiated functions (14). The cell attachment efficiency and the longevity of hepatocytes with specialized functions were also found to be improved by simultaneous addition of dexamethasone and insulin (17). Therefore, as shown in the previous report (4), the increase in the number of attached cells after a lag period seems to result from restoration of the ability of cells to repair their injured cell membranes. The membranes of isolated hepatic cells prepared by both enzymatic digestion and mechanical destruction seem to be injured, as indicated from the previous observations that they partly lost their cellular proteins such as alanine and tyrosine aminotransferases (2). Insulin may allow the injured cells to repair

their membranes and dexamethasone may give more favorable conditions of culture for their long survival. Thus, the decrease in the number of primary cultured cells (3, 5) may not be due to the nature of cultured hepatocytes but rather may result from inadequate culture conditions (17).

The epithelial cells with clear cytoplasm proliferated in medium supplemented with steroid hormones, their appearance being succesfully delayed by the combined supplementation with dexamethasone and insulin. It has been reported that the effect of hydrocortisone on maintaining the epithelial features of liver cell cultures can be demonstrated only if it is continuously present in the medium (18). Murison has already pointed out that hydrocortisone has a marked inhibitory effect on the proliferation of fibroblasts at least in the initial 2 to 3 days of the primary culture of liver cells (18). However, it is not clear whether these steroid hormones inhibit the growth of fibroblasts and change of liver epithelial cells to morphologically fibroblast-like cells or enhance the proliferation of epithelial cells. This observation that cultures containing epithelial clear cells exhibited relatively higher concentrations of albumin in the spent media supplemented with these steroids favors the latter view. The clear cells also resemble the undifferentiated cells found in the epithelial cell lines established from adult rat livers (19). Therefore, these steroid hormones might induce albumin production in the epithelial clear cells, *i. e.*, the undifferentiated liver cells in long-term culture might be also differentiated by these hormones.

Maintenance of viable hepatocytes with liver-specific functions in culture is of particular importance for undertaking experiments *in vitro* in parallel to those *in vivo*, where animal studies are generally performed for several days with various special treatments. It is expected that the short-term experiments employing rat livers as the target organ *in vivo* could be carried out in the experimental systems *in vitro* by using the improved conditions of primary culture as described in this study.

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Legends to Photographs

Photos 1 to 4. The culture-ages of Photos 1, 2, 3 and 4 are 2, 4, 6 and 8 day-old postinoculation, respectively. The final concentrations of hormones added to the control medium are as follows: a, control; b, dexamethasone disodium phosphate (1.0×10^{-5} M) and insulin (1.6×10^{-6} M); c, dexamethasone (2.5×10^{-6} M); d, hydrocortisone (3.0×10^{-6} M); e, insulin (1.6×10^{-6} M) and f, glucagon (1.6×10^{-6} M). Cultured cells were observed under a phase contrast microscope at a magnification of 100 times.

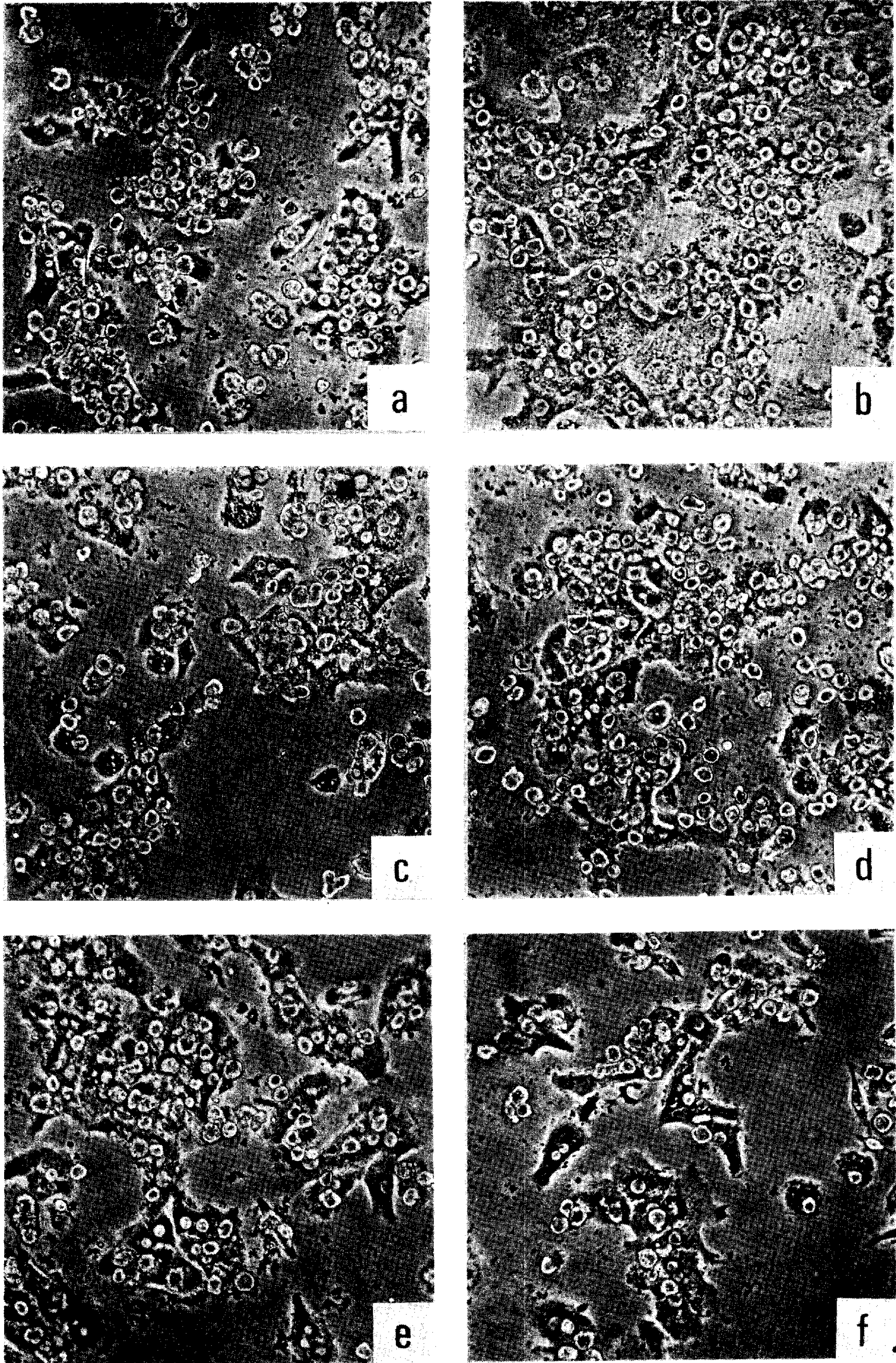


Photo 1

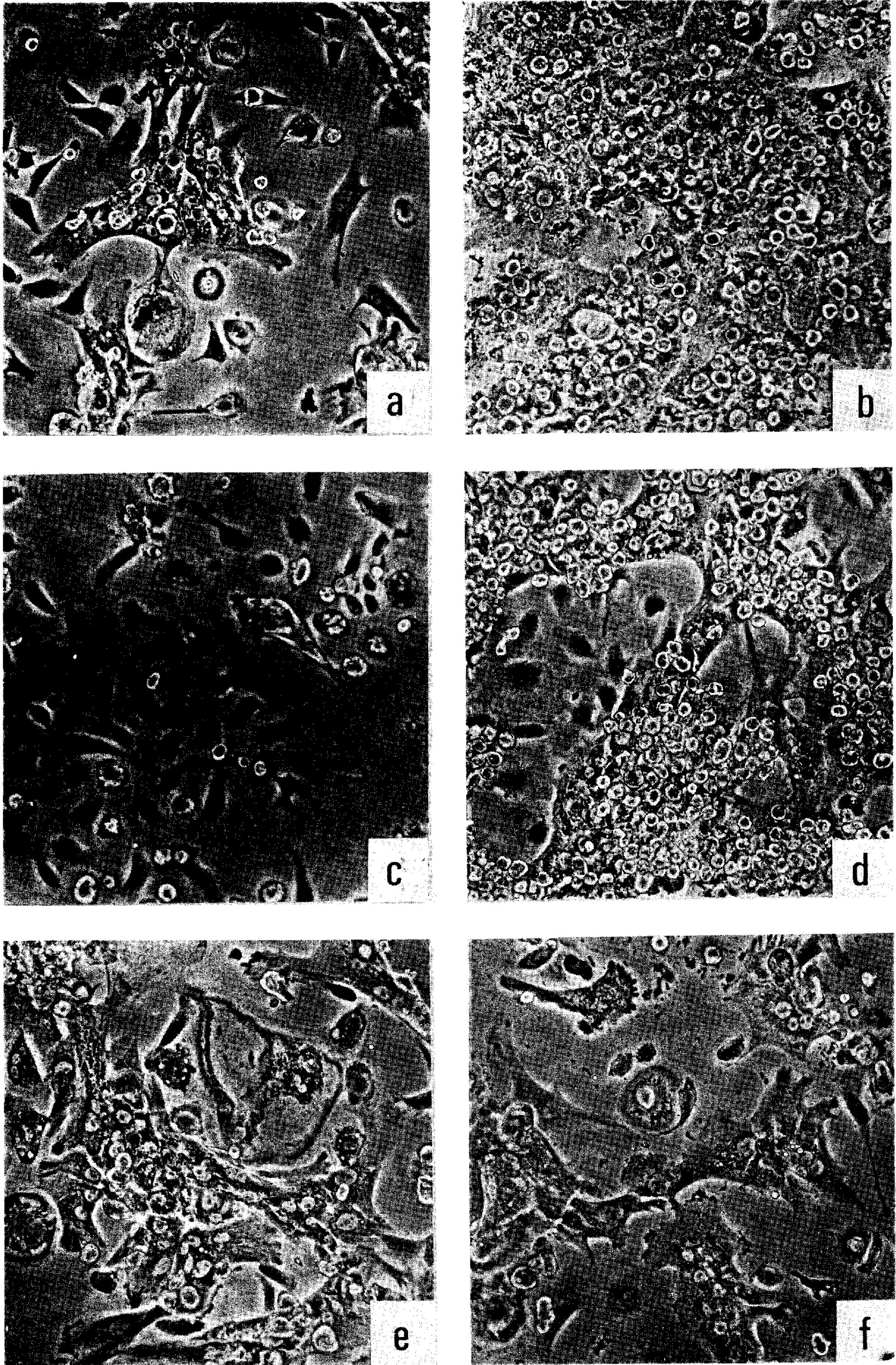


Photo 2

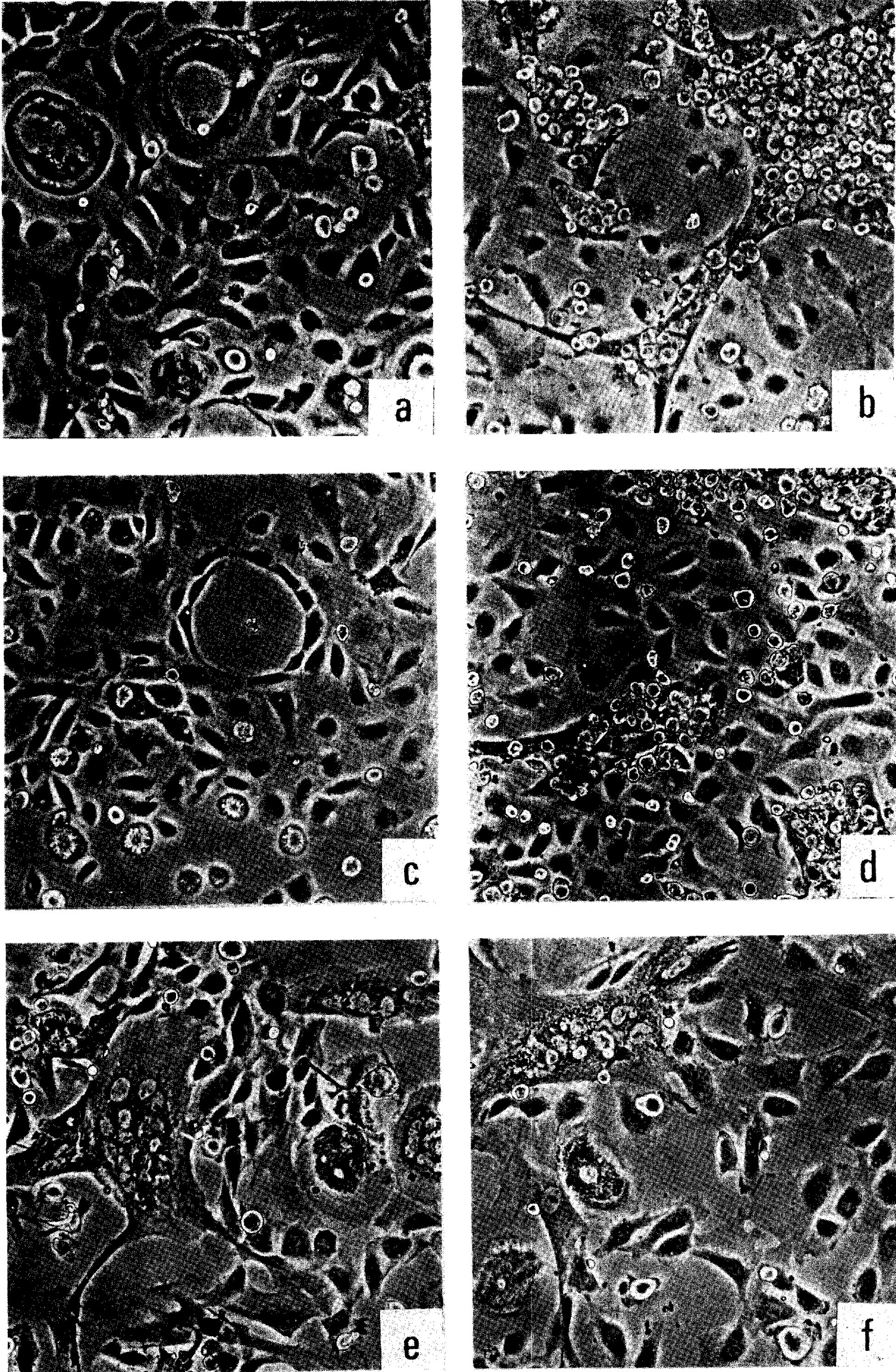
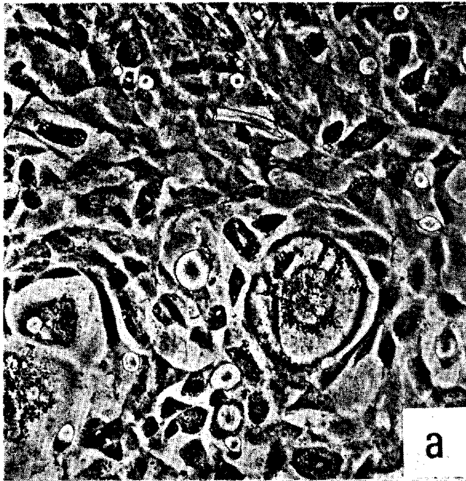
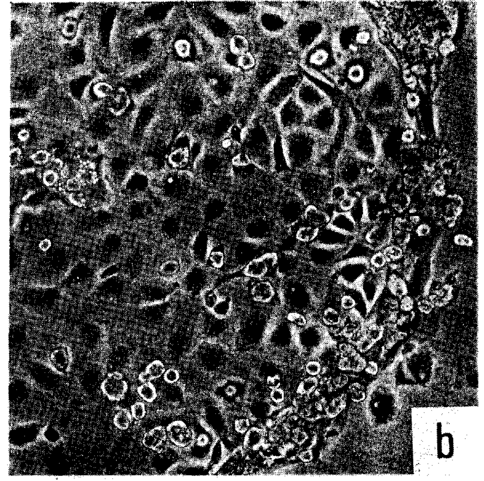
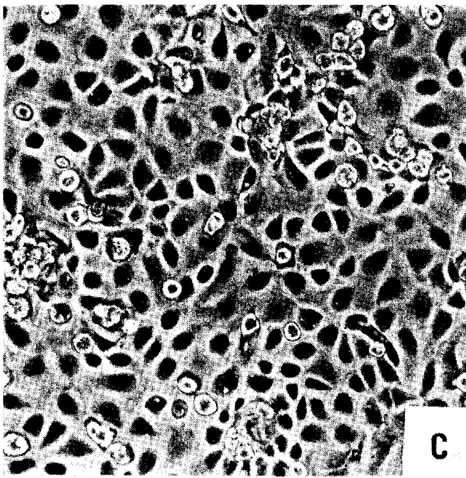
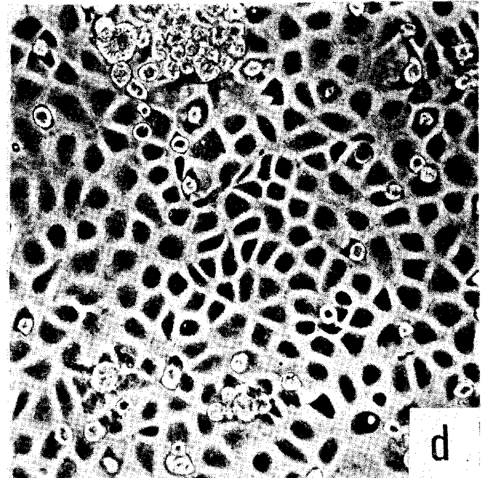


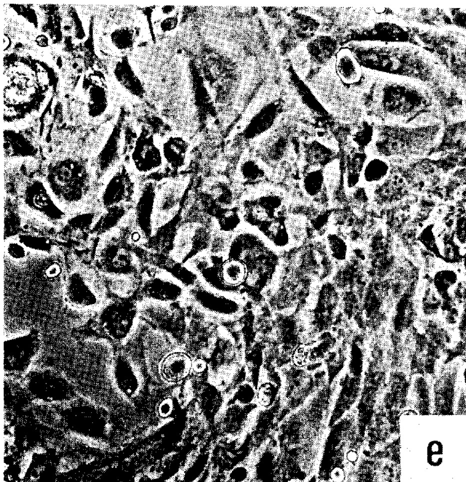
Photo 3











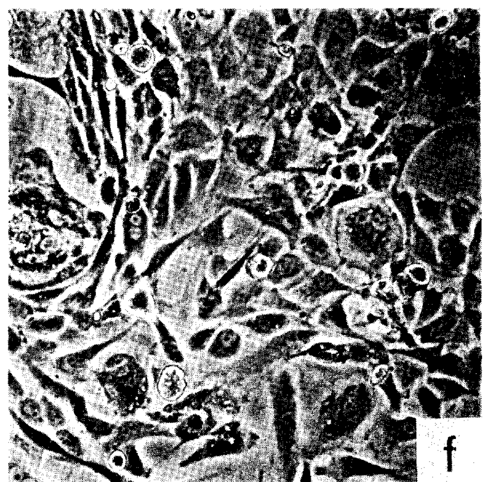


Photo 4