Effects of synthetic media and nutritional sources on in vitro maturation of bovine follicular oocytes

Effects of synthetic media and nutritional sources on *in vitro* maturation of bovine follicular oocytes

Yutaka FUKUI, Souichi ISHIDA, Yoshinori TERAWAKI and Hitoshi ONO

Department of Meat Animal Reproduction, Obihiro University of Agriculure and Veterinary Medicine Obihiro Hokkaido, Japan 080

(Received: December 23, 1980)

Abstract

The present study was undertaken to investigate effects of three synthetic media (TCM 199, Ham-F-10, BMOC-3), the addition of either 15% fetal calf serum (FCS) or 4 mg/m*l* bovine serum albumin (BSA) and the addition of sodium pyruvate (Na-P), sodium lactate (Na-L) or both for *in vitro* maturation of bovine follicular oocytes.

Oocytes were collected from follicles $\leq$5 mm in diameter and only those surrounded by the cumulus cells were cultured for 27-30 h. In total, 862 oocytes were examined for the stage of meiotic division after fixation and staining.

There were highly significant differences in the proportions of matured oocytes among the media ($P<0.001$) and between the additions of FCS and BSA ($P<0.001$). Concerning the media, BMOC-3 was superior to the other two used. There was found a high interaction between the media and the addition of either FCS or BSA ($P<0.001$). However, it was revealed that there was no significant difference for oocyte maturation between FCS and BSA when the TCM 199 medium was used. The addition of FCS to Ham-F-10 and BMOC-3 resulted in significant differences in the proportions of matured oocytes compared to the supplement of BSA ($P<0.001$). BMOC-3 supplemented with FCS showed higher proportions of matured oocytes than the other media combined with either FCS or BSA. The addition of Na-P, Na-L or both to the medium did not significantly affect oocyte maturation. However, the use of Na-P appeared to improve the rates of maturation more than without it.

The best result was obtained by the culture in BMOC-3 containing FCS and Na-P (78.6%). It has been concluded that bovine oocyte maturation was significantly altered by the media and the addition of nutritional sources such as FCS, BSA, Na-P and Na-L.

Introduction

Bovine follicular oocytes have been cultured for *in vitro* maturation by many researchers (SREENAN 1970, SHEA *et al.* 1976, SATO *et al.* 1977, TROUNSON *et al.* 1977, SATO *et al.* 1978, MEINECKE and MEINECKE-TILLMANN 1979 and FUKUI and SAKUMA 1980 a, b). Media used for culture has varied and the addition of nutritional sources such as FCS, BSA, Na-P, Na-L and others were also different

among the workers. FUKUI and SAKUMA (1980 a) previously reported the effects of three synthetic culture media (TCM 199, Ham-F-12 and Dulbecco's phosphate buffered saline: PBS) for bovine oocyte maturation *in vitro*.

The present study was undertaken to obtain further detailed information on additional effects of FCS, BSA, Na-P and Na-L in the synthetic media of TCM 199, Ham-F-10 and BMOC-3.

Materials and Methods

Ovaries were obtained from cows at a local slaughterhouse and brought to the laboratory in 0.9% saline solution at 35-37°C within 2 hours. Follicaular oocytes were collected by puncturing follicles $\leqslant$5 mm in diameter with a 22 gauge needle, regardless of the presence of corpus luteum in the ovary. Because the ovaries were obtained from a slaughterhouse, the stage of the estrous cycle of each animal was unknown.

The media used for the present study were as follows: TCM 199 (Laboratory of Microbiology, Osaka Univ, Japan), Ham-F-10 (Gibco Lab., U. S. A.) and BMOC-3 (Brinster's Medium: Gibco Lab., U. S. A.). Each medium contains either 15% FCS (Flow Lab., Australia) or 4 mg/m*l* BSA (Fraction V: Sigma Co., U. S. A.) with 50 μg/m*l* streptomycin and 100 i. u./m*l* penicillin G. To the medium was also added either 0.25 mM Na-P, 25 mM Na-L, both, or neither (control). The pH of the media was adjusted to 7.2 and sterilized by filtration through 0.45 μm millipore membrane.

The collected oocytes were carefully examined for culture and those surrounded by the cumulus cells were transferred to 1 m*l* of the medium in a test tube (60 mm long, 10 mm in diameter) kept at 37-38°C. The medium placed in the test tube had been allowed to preincubate for 2-3 h at 37-38°C after gassing with 5% CO_2 in air for 30 sec. Gassing for 1 min was again performed after transferring 5-10 oocytes to each tube. The test tubes were tightly stoppered and kept in the incubator at 37-38°C for 27-30 h. After the culture was terminated, the oocytes were denuded in 0.2% sodium citrate (HIRSCHEL and HUNTER 1979). The denuded oocytes were then fixed in acetic alchohl (1: 3) for 24-48 h and stained with 0.75% aceto-orcein. The stage of oocyte maturation was examined by a phase contrast microscopy. Classification of the maturation stage in the meiotic division was based on the method described by SATO *et al.* (1978) (Plates I and II).

The factorial design (3×2×4) was as follows: media; TCM 199, Ham-F-10, BMOC-3, the addition of either FCS or BSA and the addition of either Na-P, Na-L, both, or neither (control). The proportions of oocytes reaching the second metaphase (M-II) of the meiotic division were subjected to least squares method for analysis of variance after angular transformation (STEEL and TORRIE 1960).

Results

In total, 862 oocytes were examined for their maturation stages. The results are shown in Table 1. Out of 862, 409 oocytes (47.4%) had reached the metaphase-II stage of meiotic division after culture. By analysis of variance (Table 2), there were highly significant differences for the proportions of matured oocytes among the media ($P<0.001$) and between the media containing, FCS and BSA ($P<0.001$). There was also found to be a high interaction between the media and the addition of either FCS or BSA ($P<0.001$). However, it was revealed by the chi-square test that there was no significant difference for oocyte maturation between FCS and BSA when TCM 199 was used. The addition of FCS to the media of Ham-F-10 or BMOC-3 resulted in significantly higher rates of matured oocytes than the supplement of BSA ($P<0.001$). BMOC-3 medium was superior to the other two media and BMOC-3+FCS medium resulted in higher rates of matured oocytes than the other combined media. The proportions of matured oocytes by the medium of Ham-F-10+BSA were extremely lower than those of oocytes cultured in the other media.

The addition of Na-P, Na-L or both did not

Table 1. *In vitro* maturation of bovine oocytes cultured in different synthetic media and the addition of nutritional sources.

Medium	Addition[1] of either FCS or BSA	Addition[2] of Na-P and/or Na-L	No. of oocytes examined	Stages of maturation[3]						Degeneratives
				GV	P-I	M-I	A-I	T-I	M-II (%)	
TCM 199	BSA	Na-P+Na-L	31	0	2	6	0	3	17 (54.8)	3
		Na-P	26	1	3	4	0	2	14 (53.8)	2
		Na-L	40	0	6	6	1	5	16 (40.0)	6
		None	48	1	0	18	2	3	23 (47.9)	1
	FCS	Na-P+Na-L	30	0	3	8	0	4	13 (43.3)	2
		Na-P	39	1	4	8	0	3	22 (56.4)	1
		Na-L	34	1	5	4	0	6	16 (47.1)	2
		NOne	33	0	3	4	2	1	22 (66.7)	1
Ham-F-10	FCS	Na-P+Na-L	39	6	2	4	1	2	21 (53.8)	3
		Na-P	40	3	0	8	1	1	23 (57.5)	4
		Na-L	31	2	4	4	0	0	17 (54.8)	4
		None	45	2	3	12	0	2	24 (53.3)	2
	BSA	Na-P+Na-L	31	9	14	5	0	0	1 (3.2)	2
		Na-P	28	8	6	9	0	0	1 (3.6)	4
		Na-L	40	14	11	3	0	2	5 (12.5)	5
		None	44	10	25	2	0	1	3 (6.8)	3
BMOC-3	FCS	Na-P+Na-L	30	0	0	4	0	1	23 (76.7)	2
		Na-P	28	0	0	4	0	1	22 (78.6)	1
		Na-L	44	0	1	9	1	3	29 (65.9)	1
		None	46	1	0	11	2	2	30 (65.2)	0
	BSA	Na-P+Na-L	30	3	5	9	0	2	10 (33.3)	1
		Na-P	30	0	3	7	1	0	18 (60.0)	1
		Na-L	35	3	6	6	0	2	16 (45.7)	2
		None	40	3	4	5	1	3	23 (57.5)	1

1 FCS: 15% fetal calves serum, BSA: 4 mg/m*l* bovine serum albumin.
2 Na-P: 0.25 mM sodium pyruvate, Na-L: 25 mM sodium lactate.
3 GV: germinal vesicle, P-I: prometaphase-I, M-I: metaphase-I, A-I: anaphase-I, T-I: telophase-I, M-II: metaphase-II.

Table 2. Analysis of variance for the data of Table 1.

Source of Variation	DF	MS	F	
Media (A)	2	868.78	36.8	***
FCS or BSA (B)	1	1328.34	56.29	***
None, Na-P, Na-L or Na-P+Na-L (C)	3	26.10	1.11	
A×B	2	645.80	27.40	***
A×C	6	20.03	0.85	
B×C	3	48.78	2.07	
A×B×C	6	14.62	0.62	
Error		23.60		

*** $P < 0.001$

have significant effects on oocyte maturation, although the use of Na-P appeared to slightly improve the proportions of matured oocytes. The best results wereobtained by the culture in the medium of BMOC-3 +FCS+Na-P (78.6%).

Discussion

Various media for *in vitro* culture of bovine oocytes have been used, namely TCM 199 (SATO *et al.* 1977, 1978, LEIBFRIED and FIRST 1979 a, FUKUI and SAKUMA 1980 a), modified krebs-ringer bicarbonate solution (IRITANI and NIWA 1977, SATO *et al.* 1977, 1978), Ham-F-10 (SHEA *et al.* 1976, NEWCOMB *et al.* 1978), Ham-F-12 (SATO *et al.* 1977, 1978, FUKUI and SAKUMA 1980 a), basal eagle medium (SATO *et al.* 1977, 1978, LEIBFRIED and FIRST 1978 b), synthetic oviduct fluid (CHURCH *et al.* 1974, POPE and STEPHENS 1974, POPE and TURMAN 1974), BMOC-3 (CHURCH *et al.* 1974), growth medium (SREENAN 1970), Tyrode's solution with follicular fluid (HUNTER *et al.* 1972), Dulbecco's PBS (FUKUI and SAKUMA 1980 a) and fetal serum (TROUNSON *et al.* 1977). To compare these media for effects on *in vitro* maturation of oocytes, it would not be easy to decide on the most appropriate medium. In the previous study (FUKUI and SAKUMA 1980 a), there was no significant difference for oocyte maturation among TCM 199, Ham-F-12 and Dulbecco's PBS. However, with the addition of either 20% FCS or 5 mg/m*l* BSA, it was found that TCM 199+BSA and Ham-F-12+FCS resulted in higher rates of matured oocytes than the other combination. The present study has also shown that the combination of the basic medium and the addition of either ECS or BSA significantly affected the maturation rate ($P < 0.001$). For instance, the culture in the medium of Ham-F-10+BSA extremely depressed the resumption of meiotic division, which may be due to the deficiency of some chemical components in this combined medium.

The three types of culture media ussed in the present study, differ widely in chemical composition (TSAFRIRI and CHANNING 1975, MCGAUGHEY 1977). Nevertheless, HILLENJÖ *et al.* (1980) described that both TCM 199 and BMOC-3 supported porcine oocyte maturation equally well. The present study found that BMOC-3 medium resulted in signficantly higher rates of bovine oocyte maturation than the TCM 199 medium. It has been well established that the defined minimal culture medium (BMOC) can support oocyte maturation *in vitro* in several species (CROSS and BRINSTER 1970, GWATKIN 1972 and CHURCH *et al.* 1974). CHURCH *et al.* (1974) cultured bovine oocytes for 24 h in BMOC-3 medium and obtained a 54% maturation rate. They further found that the addition of 12 mg/m*l* of BSA to the medium increased the proportion of matured oocytes (76%). In the present study, the addition of BSA (4 mg/m*l*) to each medium was less effective on oocyte maturation, especially in the media of Ham-F-10 and BMOC-3, than the addition of 15% FCS. Considering the results of CHURCH *et al.*, (1974) and the present data, higher levels of BSA such as 12 mg/m*l* may be needed when Ham-F-10 or BMOC-3 medium was used.

BSA has been added to medium for *in vitro* maturation of bovine oocytes (CHURCH *et al.* 1974, IRITANI and NIWA 1977, SATO *et al.* 1977, 1978 and MEINECKE and MEINECKE-TILLMANN 1979). However, comparable studies on the effects of the addition of either BSA or FCS has not been attempted. The present study has shown that FCS significantly improves the rates of maturation compored to the addition of BSA, although the addition of either FCS or BSA to the TCM 199 medium did not result in a significant difference for oocyte maturation. IRITANI (1979) and TOYODA (1980) have suggested that the use of FCS would be unsuitable with respect to repeatability for higher maturation rates in the culture, indicationg that a chemically defined medium containing BSA should be used.

Although some workers (IRITANI and NIWA 1977, SATO *et al.* 1977, 1978) have added both Na-P and Na-L to media, the present study showed that the addition of Na-P, Na-L or both did not result in

a significant effect on oocyte maturation. However. it appeared that the media with Na-P produced higher rates of matured oocytes than the media with neither Na-P nor Na-L. The studies have demonstrated the necessity of Na-P for the maturation of denuded mouse oocytes (BIGGERS *et al.* 1967) and descriptions of the change in protein synthesis within the oocytes (SCHULTZ and WASSARMAN 1977). It has been further shown that the cumulus cells in an *in vitro* culture system are capapble of utilizing glucose to supply Na-P in the mouse oocytes for maturation (DONAHUE and STERN 1968). Na-P, Na-L or oxaloacetate must be added to the culture medium for the survival and development of the fertilized ovum, beccause glucose cannot be utilized until cleavage has begun at which time glucose is incorporated into mouse, monkey and bovine embryos (BRINSTER 1971 and BOONE *et al.* 1978).

For oocytes maturation, the cumulus cells surrounding the oocyte have been demonstrated to be the most important factor influenced (MCGAUGHEY 1977, SATO *et al.* 1977, 1978 and FUKUI and SAKUMA 1980 b). Oocyte maturation *in vitro* has grealy relied on the presence of the cumulus for the passage of energy sources such as proteins (SCHULTZ and WASSARMAN 1977), Na-P (BIGGERS *et al.* 1967), amino acids (MOOR and SMITH 1979), calcium and magnesium ions (LEIBRIED and FIRST 1979 b) and steroid hormones (ROBERTSON and BARKER 1969) capable of regulating maturation.

The initial thing important for successful oocyte maturation *in vitro* is careful selection of oocytes before the culture (MCGAUGHEY 1978, LEIBFRIED and FIRST 1979 a and MCGAUGHEY 1979). MCGAUGHEY (1978) grossly classified the porcine oocytes as follows: "good" if they are well rounded and are completely surrounded by a dense layer of the cumulus cells, "fair" if they are round with a nearly complete layer of surrounding cells and "poor" if they are either abnormally shaped or have few or no surrounding cells. In the present study, oocytes classified as "good" and "fair" (Fig. 1) were cultured after further selection under low magnification (approximately 20 X). Quality evaluation of oocytes and the careful elimination of obviously degenerate or denuded oocytes would be an initial step for high proportion of maturation after *in vitro* culture.

Acknowledgement

We wish to thank the Staff of the Animal Food Center, Obihiro, Japan for their interests and for supplying bovine ovaries. Mr. A. Tajima and Mrs. J. Okazaki are appreciated for their general assistance.

References

1) BIGGERS, J. D., D. G. WHITTINGHAM, and R. P. DONAHUE (1976): Proc. Natl. Acad. Sci., U. S. A., **58**: 560-567.
2) BOONE, W. R., J. F. DICKEY, L. J. LUSZCZ, J. R. DANTZLER, and J. R. HILL (1978): J. Anim. Sci., **47**: 908-913.
3) BRINSTER, R. L. (1971): J. Reprod. Fert., **24**: 187-191.
4) CHURCH, R. B., B. F. SHEA, and R. TERVIT (1974): J. Anim. Sci., **39**: 142, Abstr.
5) CROSS, P. C., and R. L. BRINSTER (1978): Biol. Reprod., **3**: 298-307.
6) DONAHUE, D., and S. STERN (1968): J. Reprod. Fert., **17**: 395-398.
7) FUKUI, Y., and Y. SAKUMA (1980a): J. Tokyo Vet. Zootech., **27**: 97-101.
8) FUKUI, Y., and Y. SAKUMA (1980b): Biol. Reprod., **22**: 669-673.
9) GWATKIN, R. B. L. (1972): In Vitro, 8: 59-67.
10) HILLENJÖ, T., A. S. KRIPNER, S. H. POMERANTZ, and C. P. CHANNING (197): In Ovarian Follicle and Corpus Luteum Function, Eds. C. P. Channing, J. M. Marsh & W. A. Sadler, pp. 283, Pleum Press, N. Y., and London.
11) HIRSCHEL, M. D., and A. G. HUNTER (1979): J. Anim. Sci., **49**: Suppl. 1: 304, Abstr.
12) HUNTER, R. H. F., R. A. S. LAWSON, and L. E. A. ROWSON (1972): J. Reprod. Fert., **30**:

325-328.
13) IRITANI, A. (1979): Personal communication.
14) IRITANI, A., and T. NIWA (1977): J. Reprod. Fert., 50: 119-121.
15) LEIBFRIED, L., and N. L. FIRST (1979a): J. Anim. Sci., 48: 76-86.
16) LEIBFRIED, L., and N. L. FIRST (1979b): J. Exp. Zool., 210: 575-580.
17) MCGAUGHEY, R. W. (1977): Exp. Cell Res., 109: 25-30.
18) MCGAUGHEY, R. W. (1978): In Methods in Mammalian Reproduction, Ed. J. C. Daniel, pp. 1, Academic Press, N. Y.
19) MCGAUGHEY, R. W. (1979): J. Exp. Zool., 209: 239-254.
20) MEINECKE, B., and MEINECKE-TILLMANN (1979): Dtsch. Tierarztl. Wschr., 86: 477-479.
21) MOOR, R. M., and M. W. SMITH (1979): Exp. Cell Res., 119: 333-341.
22) NEWCOMB, R., W. B. CHRISTIE, and L. E. A. ROWSON (1978): Vet. Rec., 102: 461-462.
23) POPE, C. E., and D. F. STEPHENS (1974): J. Anim, Sci., 38: 216, Abstr.
24) POPE, C. E., and E. J. TURMAN (1974): J. Anim. Sci., 39: 221, Abstr.
25) ROBERTSON, J. E., and R. D. BARKER (1969): Proc. 2 nd Meet. Soc. Study Reprod., Univ. Calif. Davis. pp. 29, Academic Press, N. Y.
26) SATO, E., A. IRITANI, and Y. NISHIKAWA (1977): Jap. J. Anim. Reprod., 23: 12-18.
27) SATO, E., A. IRITANI, and Y. NINSHIKAWA (1978): Jap. J. Zootech. Sci., 49: 236-242.
28) SCHULTZ, R. M., and P. M. WASSARMAN (1977): J. Cell Sci., 24: 167-194.
29) SHEA, B. F., J. P. A. LATOUR, K. N. BEDIRIAN, and R. D. BARKER (1976): J. Anim. Sci., 43: 809-815.
30) SREENAN, J. (1970): J. Afric. Sci., Camb., 75: 393-396.
31) STEEL, R. G. D., and J. H. TORRIE (1960): In Principles and Procedures of Statistics. Eds. the authors, pp. 158, McGraw-Hill Book Comp. INC.
32) TOYODA, Y. (1980): Shusanki-Igaku, 10: 43-48.
33) TROUNSON, A. O., S. M. WILLADSEN, and L. E. A. ROWOSON (1977): J. Reprod. Fert., 51: 321-327.
34) TSAFRIRI, A., and C. P. CHANNING (1975): Endocrinology, 96: 922-927.

ウシ卵胞内卵子の体外成熟におよぼす合成培養液と栄養源の影響

福井豊・石田聡一・寺脇良悟・小野斉
(帯広畜産大学肉畜増殖学研究室，帯広市稲田町 080)

摘　　要

ウシの卵胞内卵子（卵母細胞）を直径 5 mm 以下の卵胞から取り出し，体外成熟に影響する要因として3種類の合成培養液（TCM 199, Ham-F-10, BMOC-3）と添加栄養素について検討した。添加剤としては各々の培養液に15% 牛胎児血清（FCS）または 4 mg/ml 牛血清アルブミン（BSA）を，さらに 0.25 mM ピルビン酸ナトリウム（Na-P），25 mM 乳酸ナトリウム（Na-L），この両者，または無添加で培養後（27-30 h）の成熟（第二成熟分裂の中期）した卵子の割合を比較し，次のような成績を得た。

1) 成熟卵子の割合について，使用した培養液間（$P<0.001$）および FCS と BSA 間（$P<0.001$）に高い有意差が認められた。使用した3種の培養液の内 BMOC-3 液が最も良好であるように思われた。

2) 培養液と FCS または BSA 間に高い相互作用（$P<0.001$）が認められた。すなわち，Ham-F-10 または BMOC-3 液を使用した場合は FCS を液加した方が有意に高い成熟率を示したが，TCM 199 を使用した場合は FCS と BSA 間に有意差は認められなかった。

3) Na-P, Na-L, Na-P+Na-L または無添加区において卵子の成熟率に有意差は見られなかったが，Na-P を添加した場合は無添加区よりも良好であるように思われた。

4) BMOC-3 液に FCS と Na-P を添加した場合に最高の卵子成熟率（78.6%）が得られた。

Explanation of Plates

Plate I

Fig. 1. Bovine follicular oocytes with and without the cumulus cells (non-stained, X 200). Only the oocytes surrounded by the cells were cultured for 27–30 h in the present study.

Fig. 2. After the culture, an oocyte has the germinal vesicle, which did not resume the meiotic division at all (non-stained, X 400).

Fig. 3. Metaphase I of an oocyte maturing *in vitro* (stained with aceto-orcein, X 1000).

Plate II

Fig. 4. Anaphase I of an oocyte maturing *in vitro* (stained with aceto-orcein, X 1000).

Fig. 5. First polar body (lower-right) and second metaphase (upper-left) are seen (stained with aceto-orcein, X1000).

Fig. 6. First polar body released from the ooplasm is clearly seen (non-stained, X 400).

Plate I

Fig. 1.

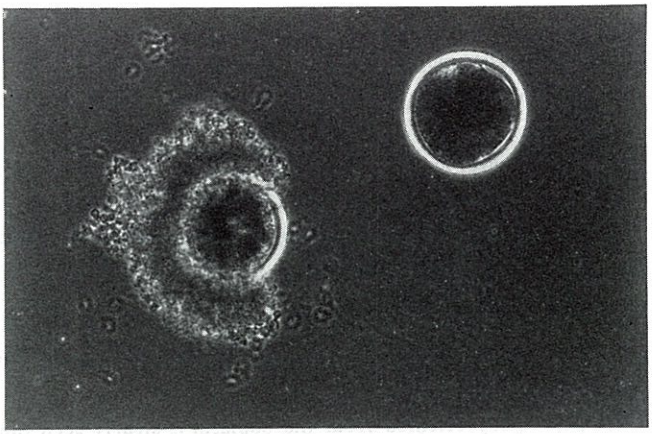

Fig. 2.

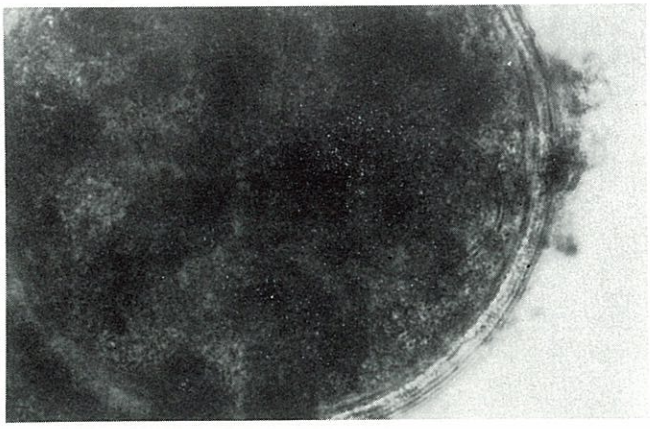

Fig. 3.

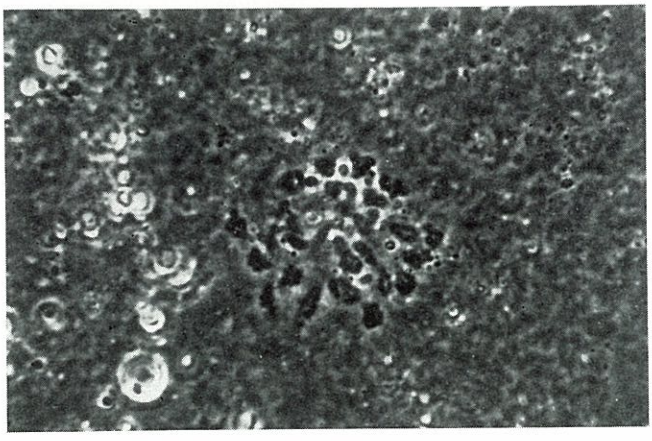

Plate II

Fig. 4.

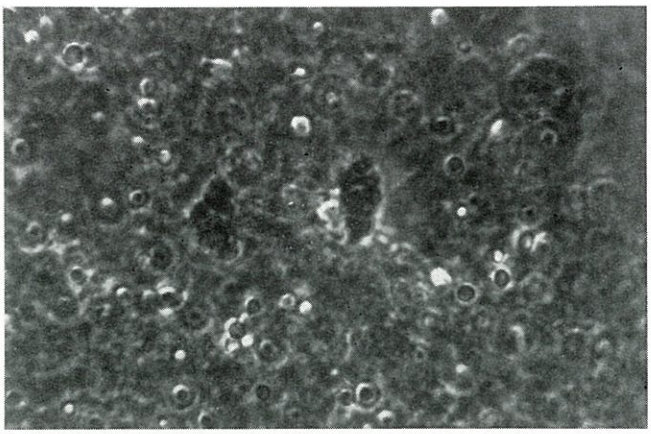

Fig. 5.

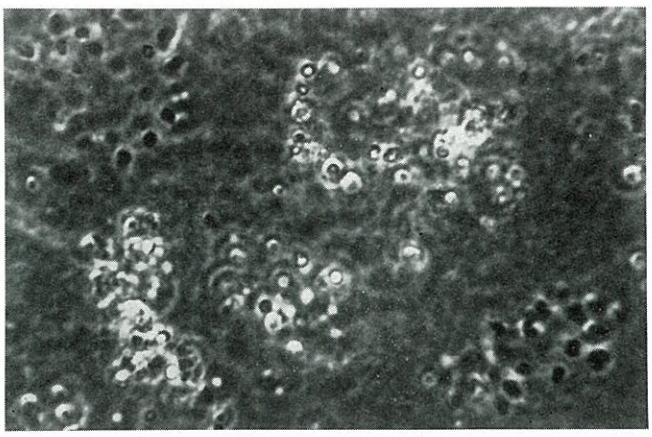

Fig. 6.