

CYTOTOXIC EFFECTS OF A NITROFURAN DERIVATIVE ON AN ASCITES CARCINOMA CELLS

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ABSTRACT

The effects of Panfuran-S on the growth of Ehrlich ascites carcinoma cells have been studied.

Marked cytotoxic effects were observed in the experiments in which the tumor cells were incubated with the drug.

In contrast to the effects on the tumor cells *in vitro*, treatment with the drug failed to inhibit tumor growth in Swiss ICR/Ha mice receiving injection of the tumor cells whether it was administered by the subcutaneous, intrabdominal, intratumorous, or oral route.

This drug was also toxic to human fibroblast cells in tissue culture study.

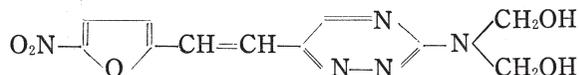
INTRODUCTION

In the search for new antibacterial drugs, over hundreds of nitrofurantoin derivatives were synthesized; incidentally, their antitumor properties were found in rodents and man.

Furacin (5-nitro-2-furaldehyde semicarbazone) has first been reported to delay the growth of a transplanted fibrosarcoma in mice¹⁾. Furadroxyl (5-nitro-3-furaldehyde 2-(2-hydroxyethyl) semicarbazone) has then been reported to have a specific degenerative effect upon the testicular germinal epithelium in rats²⁾.

These findings led to clinical application, mainly by urologists, indicating that these drugs were effective in the treatment of testicular tumors³⁾⁻⁵⁾. Extensive studies on nitrofurantoin derivatives were performed by Miura, *et al.*⁶⁾⁻⁸⁾.

Recently, a new nitrofurantoin derivative, 3-di(hydroxymethyl) amino-6-(5-nitro-2-furyl)ethyl-1, 2, 4-triazine (Panfuran-S),



has been synthesized; its antibacterial action is reported to be much effective than other nitrofurantoin derivatives⁹⁾.

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This report is concerned with the effects of this drug on Ehrlich ascites carcinoma cells.

MATERIALS AND METHODS

1. Compound

Panfuran-S was prepared for injection by suspending finely powdered crystals in Polyethylene Glycol in physiological saline or in Hanks balanced salt solution.

2. Animal

Male Swiss ICR/Ha mice weighing 25-28 g were employed.

3. Preparation of Ehrlich carcinoma cell suspension

Ascitic cells maintained in Swiss ICR/Ha mice were obtained by harvesting after 7 days' intraperitoneal growth. Then the tumor cells were washed three times with Ringer solution. The viable cells were counted by staining with 0.01% Trypan Blue solution. The suspension was adjusted to contain a proposed number of cells per ml.

4. Schedule of treatment

a) *Subcutaneous administration*: Mice were inoculated with the 10^6 viable cells in 0.1 ml suspension intraperitoneally and treated by subcutaneous administration of Panfuran-S starting immediately after the inoculation and continuing once daily for 7 successive days. The daily dose of the drug given were 7 mg of Panfuran-S that were equivalent to one-tenth of LD_{50} of mouse. Control mice received the same amount of physiological saline or Polyethylene Glycol as used for dosing. Excluding dead animals without tumor development, survival time of inoculated mice was recorded.

b) *Oral administration*: Mice were inoculated with 10^6 viable cells intraperitoneally and treated forcibly by oral administration of the drug suspended in saline through the Nelaton tube in a daily dose of 7 mg for 3 successive days, starting on the day of inoculation. Excluding the dead mice without tumor development, survival time of mice was recorded.

c) *Intraperitoneal administration*: Mice were inoculated with 10^6 viable cells intraperitoneally and treated with intraperitoneal administration of the drug suspended in saline in a daily dose of 1 mg for 4 successive days starting on the 7th day when ascites developed. The survival time of mice was recorded.

d) *Administration into tumor area*: mice were inoculated with 10^6 viable cells into the right axillary space. Nine days later when all mice developed subcutaneous tumors approximately 12 mm in diameter, the tumor area was infiltrated with 1 mg of the drug in a volume of 0.1 ml once daily for 4 successive days. Control mice received the same amount of saline. All mice were

sacrificed on the 30th day after tumor inoculation. The tumor was weighed and measured with calipers in two diameters. The volume of tumor was calculated by increased volume of water putting tumor into known volume.

5. *In vitro* study

a) *Determination of cell viability by intraperitoneal injection*: Two ml of the cell suspension containing 5×10^6 viable Ehrlich ascites carcinoma cells were incubated with 1 mg of Panfuran-S in Hanks balanced salt solution with 0.5% Lactalbumin hydrolysate for 1, 3, and 6 hours at 37°C in an incubator. After the incubation, 0.1 ml of this mixture (2.5×10^6 cells) were given by intraperitoneal injection into groups of 15 each.

b) *Determination of cell viability by dye-exclusion test*: After the incubation, this mixture, as described in the preceding paragraph, was centrifuged and washed with Ringer solution. The stained cells were counted by mixing 0.01% Trypan Blue solution.

c) *Determination of cell viability by Succinic Dehydrogenase Inhibition Test (SDI-test)*: Method of this test has been introduced in our early publications¹⁰⁾¹¹⁾. After the incubation, 0.5 ml of TTC solution consisted of 30 mg of 2, 3, 5-tripheyltetrazolium chloride and 2.7 g of sodium succinate in 50 ml of M/15 phosphate buffered saline were added to the mixture. It was further incubated for 5 hours and 4 ml of ethyl acetate containing 0.5% trichloroacetic acid was added to each mixture. Quantity of formed formazan was measured by spectrophotometer at 480 m μ .

d) *Determination of cell viability by growth rate in tissue culture*: After 3 hours' incubation, the mixture was washed and maintained in Hanks balanced salt solution with 0.5% Lactalbumin hydrolysate in an incubator. The cell counts were performed on the 1st, 2nd, and 4th day using the haemocytometer.

e) *Effect of Panfuran-S on normal cells*: A human fibroblast cells maintained in Y. L. E. medium with 20% calf serum and 10% Tryptose Phosphate Broth dehydrate (Difco) were cultivated with 500, 250, and 100 μ g per ml of Panfuran-S. The cell counts were performed on the 2nd, 4th, and 6th day.

RESULTS

Subcutaneous injection of Panfuran-S in a daily dose of 7 mg was toxic for mice and some of them resulted in an early death. All mice given Panfuran-S developed diarrhea and a progressive loss of weight in which 10 died from emaciation. The remaining 5 developed ascites.

As shown in Table 1, loss of weight was observed in Panfuran-S treated mice. Polyethylene Glycol or physiological saline injected mice gained weight due to development of ascites. Five mice which tolerated toxicity of the drug

TABLE 1. Effect of Subcutaneous Injections of Panfuran-S on Body Weight and Survival Time of Mice Bearing Ehrlich Ascites Carcinoma Cells

	Av. Body Wt. Change (g)	No. Mice Alive		
	1st week	8th day	10th day	14th day
Panfuran-S	-0.9	12/15	5/15	2/15
Polyethylene Glycol	+5.0	15/15	15/15	8/15
Physiological saline	+4.7	15/15	12/15	8/15

also developed ascites, therefore, loss of weight in Panfuran-S treated mice was remarkable.

Excluding the dead mice from toxicity of the drug, the effects of subcutaneous injection of Panfuran-S upon the growth of intraperitoneally injected Ehrlich ascites carcinoma cells are shown in Fig. 1. All treated mice tolerated toxicity and all the two groups of control mice died within 24 days after tumor inoculation as a result of malignancy. Here, Panfuran-S appears to be ineffective on Ehrlich ascites carcinoma cells regardless of high doses as to show toxicity and in this route of administration.

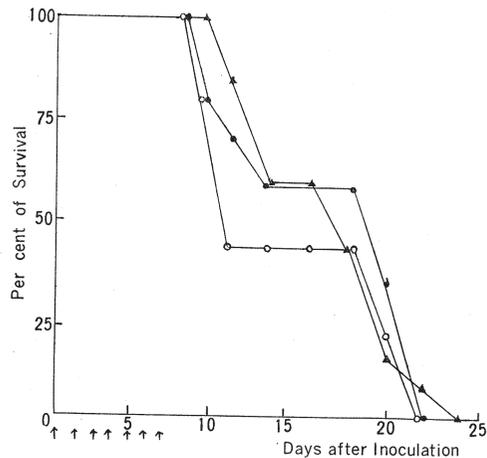


FIG. 1. Per cent survival of mice bearing Ehrlich ascites carcinoma treated with subcutaneous injections of Panfuran-S and Controls. \circ = Panfuran-S; \blacktriangle = Polyethylene Glycol; \bullet = Physiological saline; \uparrow = administration of drug.

Since subcutaneous route was largely absorbed though small amounts of the drug were observed at necropsy at the site of injection, oral administration of the drug was performed following intraperitoneal injection of Ehrlich ascites carcinoma cells. Under these conditions, 5 mice died: 2 from pneumonia and 3 from toxicity of drug. The remaining 10 mice treated with Panfuran-S and

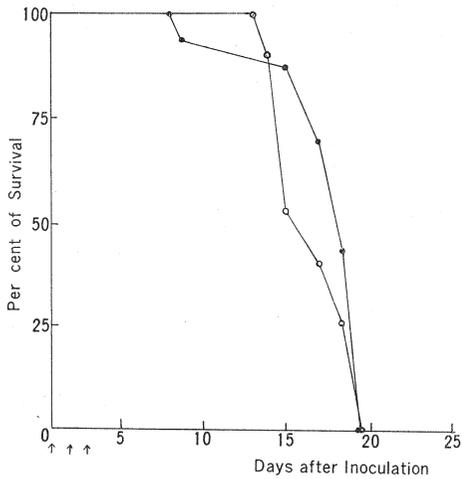


FIG. 2. Per cent survival of mice bearing Ehrlich ascites carcinoma treated with oral administrations of Panfuran-S and controls. ○=Panfuran-S; ●=Physiological saline; ↑=administration of drug.

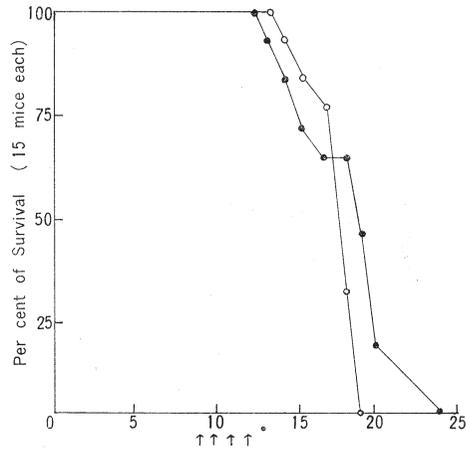


FIG. 3. Per cent survival of mice bearing Ehrlich ascites carcinoma treated with intraperitoneal administrations of Panfuran-S and controls. ○=Panfuran-S; ●=Physiological saline; ↑=administration of drug.

all the control mice died as a result of development of ascites. It can be noted from Fig. 2 that the survival rates of Panfuran-S treated mice were similar to those of control mice.

These two systemic treatments proved unsuccessful in prolongating the life span of tumor-bearing mice, therefore, two series of experiments were intended to study on topical treatment. The survival rates of the mice given Panfuran-S intraperitoneally after development of ascites were shown in Fig. 3. There was no difference in survival rates between the treated and control mice.

Similar results were recorded in the experiment in which Panfuran-S was injected directly into the subcutaneous growing tumor. It can be noted from Table 2 that the topical treatment did not prevent extensive tumor growth in subcutaneous tissue.

Although neither systemic nor topical treatments were effective, marked

TABLE 2. Effect of Panfuran-S Injected into Tumor Area on the Growth of Subcutaneously Implanted Ehrlich Ascites Carcinoma Cells, Measured on the 30th Day.

	No. mice	Av. tumor size (mm)	Total tumor weight (g)	Total tumor volume (ml)
Panfuran-S	8	26.6 × 26.0	34.14	37.0
Saline	8	22.6 × 23.9	28.74	28.0

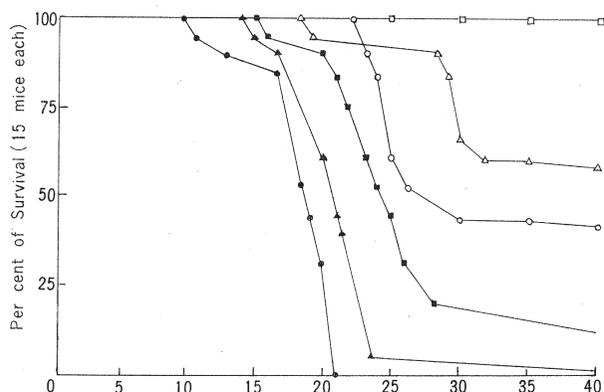


FIG. 4. Per cent of survival of mice implanted with Ehrlich ascites carcinoma cells after incubation with Panfuran-S *in vitro* and corresponding controls. □ = Panfuran-S treated for 6 hours; △ = Panfuran-S treated for 3 hours; ○ = Panfuran-S treated for 1 hour; ■ = Controls for 6 hours; ▲ = Controls for 3 hours; ● = Controls for 1 hour.

cytotoxic effects of the drug were observed in *in vitro* experiments. After incubation of Ehrlich ascites carcinoma cells with Panfuran-S for 1, 3, or 6 hours, the effects of the drug on the survival of mice given inoculation of the tumor cell suspensions are shown in Fig. 4. It is evident that the survival times prolonged as time of incubation went on. When the tumor cells were incubated with the drug for 6 hours, complete suppression of tumor development took place. With 3 hours' incubation there was pronounced effect observed but some of the mice developed tumor. In the group incubated for 1 hour, the life span produced a slow-down but it was found to be superior to those of control groups.

As indicated in Table 3, percentage of the tumor take was also related to

TABLE 3. Effects of Panfuran-S Incubated with Ehrlich Ascites Carcinoma Cells on the Incidence of Tumor Growth, Survival of Implanted Mice, Dye Staining of the Cells, and Succinic Dehydrogenase Activity of the Cells

Group	Time of incubation (Hours)	No. of mice	No. of take	50% survival (Days)	Trypan Blue Staining			SDI Test* (-Log T absorbance)
					Unstained Range(%)	Stained Range(%)	Unstained Mean(%)	
Panfuran-S	1	15	10	28.5	90-92	8-10	91.0	0.539
	3	15	9	74.0	89-93	7-11	91.3	0.405
	6	15	0		90-94	6-10	92.0	0.370
Control	1	15	15	18.2	90-94	6-10	91.3	0.515
	3	15	15	22.7	81-87	13-19	83.8	0.528
	6	15	14	25.5	40-54	46-60	44.0	0.327

*See "Materials and Methods"

the time of incubation in which 67%, 60% and nothing of tumor take occurred in the groups after 1, 3, and 6 hours' incubation, respectively.

To differentiate between viable and non-viable cells, vital staining procedure was performed by using Trypan Blue solution. Because of selective permeability, viable cells remain colorless, whereas dead cells became diffusely stained. The results were unrelated to those of bioassay experiment. This reason may be that Panfuran-S alters the character of cell membrane. Microscopically, almost all the cells treated with Panfuran-S became small and irregular, and cell membranes thick and rugged as shown in Photos. 1, 2, 3, and 4.

With dehydrogenation system, the color change was somewhat related to the results of bioassay experiment as shown in Table 3. If a dye is added to a suspension of living cell, the dye is reduced, thus revealing the percentage of viability of cell by the grade of reduction.

The effects of Panfuran-S on the tumor cells and normal cells growing in tissue culture were studied further. The number of Ehrlich ascites carcinoma cells treated with Panfuran-S reduced remarkably with the lapse of day and almost nothing on the 4th day as shown in Table 4. This effects were also observed on normal cells as shown in Table 5 and in Photos. 5, 6, 7, and 8. The untreated cells propagated, whereas, the Panfuran-S treated cells disappeared and cytoplasm were destroyed completely on the 7th day of cultivation.

TABLE 4. Effect of Panfuran-S on Ehrlich Ascites Carcinoma Cell Population in Culture

	No. cell per ml			
	Inoculation	1st day	2nd day	4th day
Panfuran-S	10×10^4	2×10^4	3×10^4	10×10^4
Controls	18×10^4	12×10^4	20×10^4	16×10^4

TABLE 5. Effect of Panfuran-S on Human Embryo Fibroblast Cell Population in Culture

	No. cell per ml			
	Inoculation	2nd day	4th day	6th day
500 μ g Panfuran-S	4×10^4	1.5×10^4	2.0×10^4	0
250 μ g Panfuran-S	4×10^4	1.5×10^4	1.5×10^4	0
100 μ g Panfuran-S	4×10^4	3.0×10^4	2.5×10^4	0
Controls	4×10^4	6.5×10^4	7.5×10^4	11×10^4

DISCUSSION

From the results obtained with Ehrlich ascites carcinoma, it is evident that Panfuran-S has cytotoxic properties *in vitro*, whereas it has no appreciable

tumor-inhibitory effect *in vivo*. Because of the fact that the tumor growth was inhibited only when the tumor cells were contacted with the drug *in vitro*, however, there was no prolongation of life span when the drug was given after tumor inoculation.

Effectiveness of nitrofuran derivatives on transplanted Ehrlich ascites carcinoma was reported by many and the relationship between chemical structure and antitumor activity was investigated. Miura, *et al.*⁹⁾, reported that among nitrofuran-quinoline derivatives, the compounds having N-oxide, carbonamide, or two amino groups on the quinoline nucleus were effective. Recently, Katae, *et al.*,¹²⁾ reported that pyrimidine, quinoline, benzimidazole, benzpyrimidine, and pyridine derivatives were effective suggesting that a nitro group on the furan nucleus might be necessary to confer antitumor activity on nitrofuran derivatives, but a furan nucleus itself might not be effective.

Thereafter, a new nitrofuran derivative, Panfuran-S, has been synthesized and Miura, *et al.*⁹⁾ observed inhibitory effect of tumor growth with Ehrlich ascites tumor following intraperitoneal administration of the drug. In the results of this study, however, there was no difference between the control and treated animals. Possibly the time of administration considers to be too late to be effective on. Nevertheless, we can not think that Panfuran-S is effective on transplanted Ehrlich ascites tumor because of negative results obtained by various routes of administration. As to this reason, one might think as suggested by Endo¹³⁾ that Panfuran-S is inactivated in liver and sufficient concentrations of drug do not reach to the tumor area. In this study, the topical uses of the drug were also ineffective.

Panfuran-S is insoluble in water, quite unstable, and sensitive to alkali and acid. Accordingly, in each experiment we used the drug immediately after the preparation. As to solvent, at first we dissolved it in Polyethylene Glycol and later in saline, since toxicities of the drug appeared to be the same between the two solutions. Concerning the pH, saline used in this study was 5.4 and this level of pH had affect on the cells when the time of incubation was prolonged. Therefore, in *in vitro* studies, we maintained the pH in the physiological range.

Although Panfuran-S had marked cytotoxic effects on the neoplastic cells *in vitro*, it was cytotoxic on normal cells as well. Consequently, the clinical use of Panfuran-S is questionable to destroy malignant cells without side effects because of the slight biochemical differences between normal and neoplastic tissue.

ACKNOWLEDGMENT

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REFERENCES

- 1) Green, M. N. and Friedgood, C. E., Action of furacin in delaying growth of a transplanted fibrosarcoma in mice, *Proc. Soc. Exp. Biol. Med.*, **69**, 603, 1948.
- 2) Prior, J. T. and Ferguson, J. H., Cytotoxic effects of a nitrofuran on the rat testis, *Cancer*, **3**, 1062, 1950.
- 3) Friedgood, C. E., Danza, A. L., and Boccabella, A., The effects of nitrofurans on the normal testis and on testicular tumors (Seminoma), *Cancer Res.*, **12**, 262, 1952.
- 4) Politano, V. A., Leadbetter, G. W., and Leadbetter, W. F., Use of furacin in treatment of testicular tumors; A case report, *J. Urology*, **79**, 771, 1958.
- 5) Szczukowski, M. J., Daywitt, A. L., and Ehrlich, H. J., Metastatic testicular tumor treated with nitrofurazone; report of a case, *J.A.M.A.*, **167**, 1066, 1958.
- 6) Miura, K., Ikeda, M., Oohashi, T., Okada, I., and Igarashi, Y., Chemical and chemotherapeutical studies on the furan derivatives. XXXIV. Antitumor activity of nitro-furanquinoline derivatives., *J. Pharm. Soc. Jap.*, **84**, 341, 1964 (in Japanese).
- 7) Miura, K., Ikeda, M., Oohashi, T., Okada, I., and Igarashi, Y., Chemical and chemotherapeutical studies on the furan derivatives. XXXV. Relationship of antitumor effect between chemical structure of nitrofurans derivatives, *J. Pharm. Soc. Jap.*, **84**, 537, 1964 (in Japanese).
- 8) Miura, K., Ikeda, M., Oohashi, T., Igarashi, Y., and Okada, I., Chemical and chemotherapeutical studies on the furan derivatives. XXXVIII. Synthesis and antitumor effect of methylol derivatives of nitrofuryl-vinyl-aminoheterocyclics, *Chem. Pharm. Bull.*, **13**, 529, 1965.
- 9) A Summary of Panfuran-S. In *Panfuran-S Bunken Syu*. p. 3, Toyama Chemical Co., Ltd., Tokyo, Japan (in Japanese).
- 10) Kondo, T., Ichihashi, H., Imamura, T., and Ohkubo, K., A new *in vitro* test of sensitivity of tumors to carcinostatic agents., *Jap. J. Cancer Clin.*, **10**, 17, 1964 (in Japanese).
- 11) Kondo, T., Imamura, T., and Ichihashi, H., *In vitro* test for sensitivity of tumor to carcinostatic agents, *Gann*, **57**, 113, 1966.
- 12) Katae, H., Iwana, H., Takase, Y., and Shimizu, M., Antitumor activity of nitrofurans and nitrothiophene derivatives on Ehrlich ascites carcinoma, *Arzneim. Forsch.*, **17**, 1030, 1967.
- 13) Endo, H., Screening of carcinostatic agents, *Jap. J. Cancer Clin.*, **11**, 105, 1965 (in Japanese).

EXPLANATION OF PHOTOGRAPHS

- PHOTO. 1. Ehrlich ascites carcinoma cells incubated with Panfuran-S for 3 hours. Cell membrane was irregular and thicker.
- PHOTO. 2. Untreated Ehrlich ascites Carcinoma cells for comparison to Photo. 1.
- PHOTO. 3. Ehrlich ascites carcinoma cells cultivated for 2 days after incubation of the cells with Panfuran-S. The cell became much smaller and spore formation.
- PHOTO. 4. Untreated Ehrlich ascites carcinoma cells cultivated for 2 days.
- PHOTO. 5. Human fibroblast cells cultivated with 500 μg of Panfuran-S for 6 days. Cytoplasm disappeared completely.
- PHOTO. 6. Human fibroblast cells cultivated with 250 μg of Panfuran-S for 6 days. Cytoplasm disappeared.
- PHOTO. 7. Human fibroblast cells cultivated with 100 μg of Panfuran-S for 6 days. Cytoplasm also disappeared.
- PHOTO. 8. Untreated human fibroblast cells showing well growing.

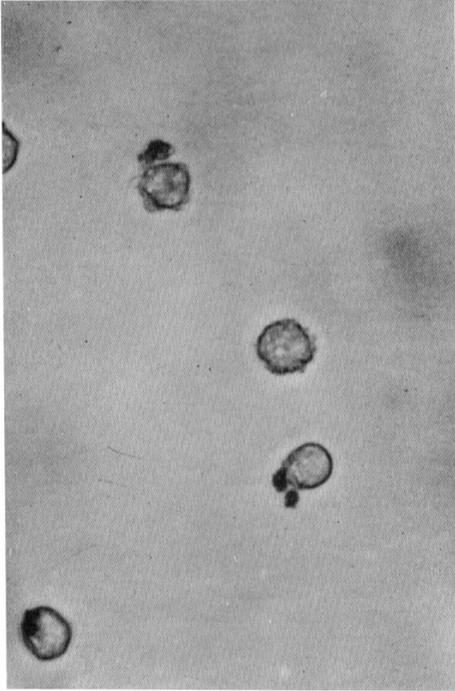


PHOTO. 1

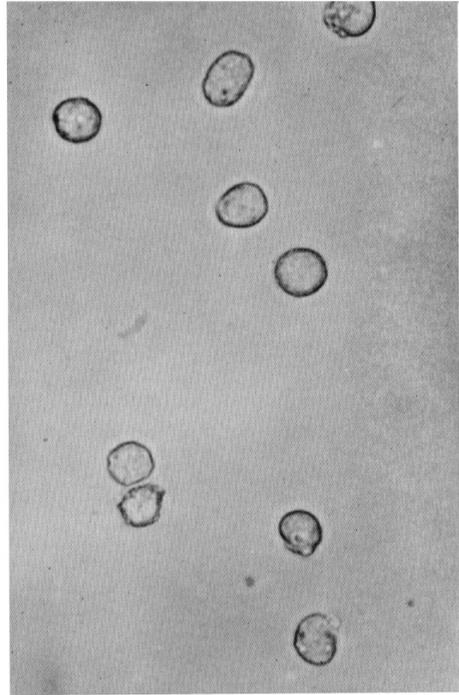


PHOTO. 2

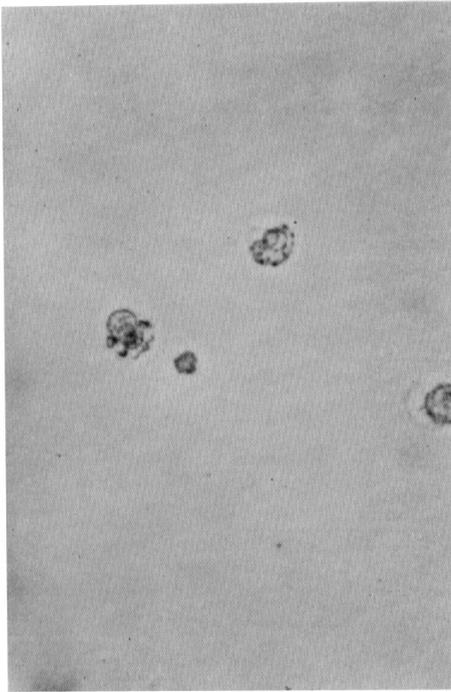


PHOTO. 3

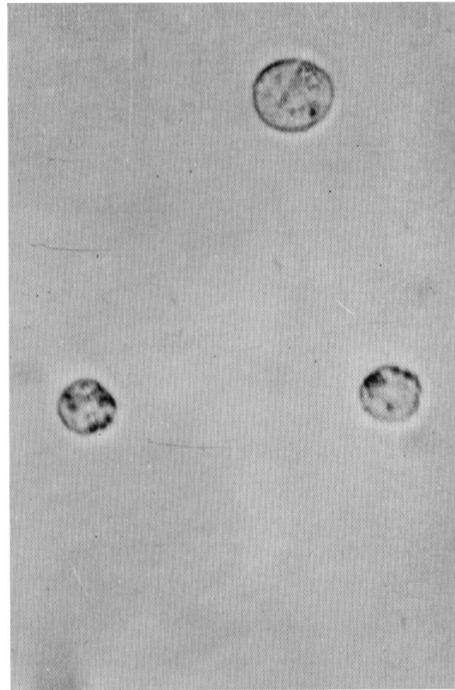


PHOTO. 4

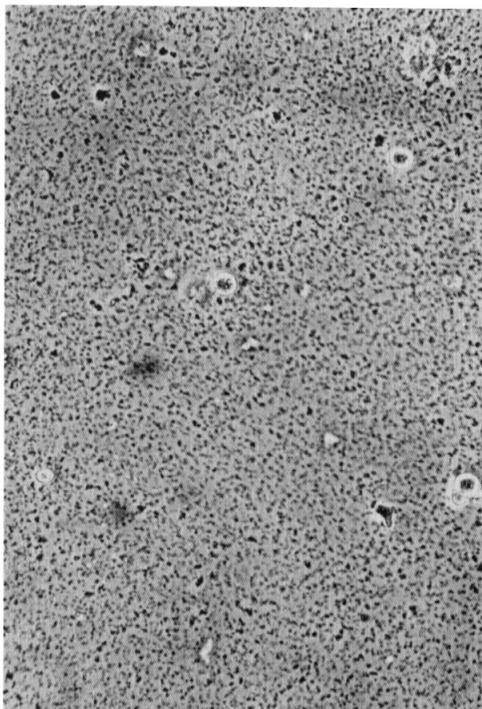


PHOTO. 5

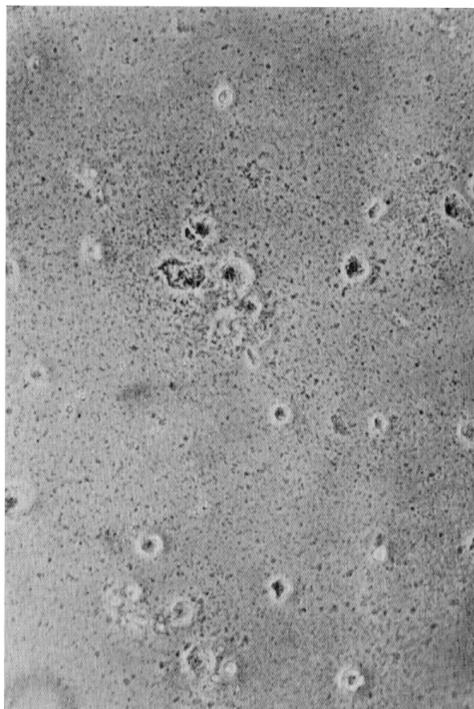


PHOTO. 6

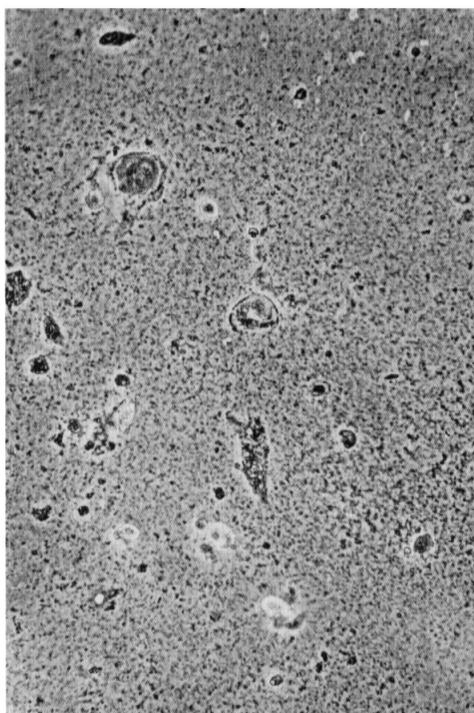


PHOTO. 7

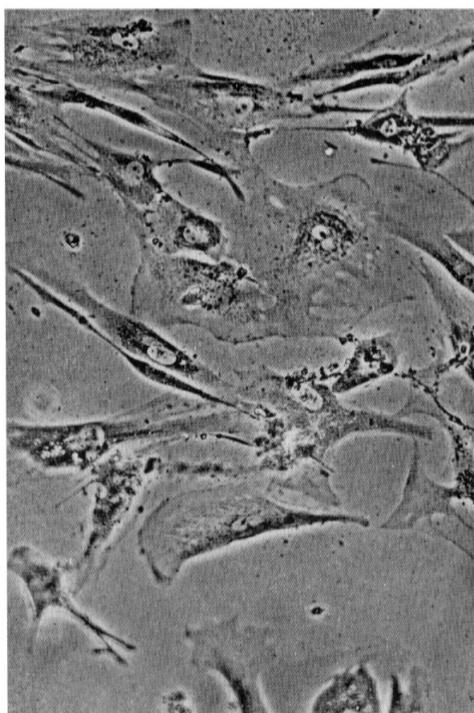


PHOTO. 8