CYTOLOGY and GROWTH

of

NORMAL and MALIGNANT

TISSUES

by

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A Thesis Submitted to the Faculty

of Graduate Studies and Research, McGill University,

in Partial Fulfillment of the Requirements

for the Degree of

Master of Science.

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1. INTRODUCTION

Several cases of tumours of the frog have been reported the most frequent being in the kidney of Rana pipiens. This tumour, which is an adenocarcinoma of unknown etiology, was first studied systematically by Lucke (1934) who in a series of papers reported the frequency, manner of growth, and other features.

The normal incidence of the tumour is about two percent in frogs that are supplied from Vermont State and from Eastern Canada, However, in the course of observations conducted in the Department of Zoology, a part of one shipment of frogs kept in a separate tank showed a much higher incidence of the tumour. The kidney of the frog is inhabited by a great number of parasites of which trematodes and myxosporidia are the most frequently occurring; the significance of the parasites in the tumour causation is not known.

Tissue culture technique offers an opportunity to conduct investigation of the behaviour of a specific part of an organ quite independant of the organism. Specific experimental conditions that cannot be applied to the organism can be maintained, controlled and reproduced. Metabolic activity, physiological nature and the response of cells to definitely known environment can be studied directly.

The recent Public Health report on Fundamental Cancer Research (1939) stressed the research objectives in the field of cancer. It was pointed out that there are two fundamental problems involved: One, the causal aspect of the cancer problem; the other the formal genesis - the the factors responsible for the nature of the cancer cells and their

uncontrolled growth which are inherent in all types of malignant cells regardless of the cause. Thus, the fundamental problem of cancer is directly associated with an understanding of the processes of growth, the metabolism and functions of normal cells.

It is with this broad aspect of the general cancer problem in mind that the investigation of behaviour of normal and malignant cells of the frog in vitro was undertaken.

The following are the main objectives of the investigation:

1. To obtain a medium best suited to the growth of normal and malignant tissues, and observations of comparative shapes and manner of growth under similar conditions.
2. To obtain pure cultures of normal kidney epithelium and compare their metabolic properties in response to respiratory poisons, cobra venom and various extracts with those of pure cultures of malignant cells.
3. To study the cytological characters of normal and malignant cells, their reaction with other cells in the colonies, the mechanism of tissue formation and cell proliferation.
4. To investigate tolerence to high and low temperature, toxicity, hydrogen ion concentration, permeability and other factors.

II. REVIEW OF LITERATURE

Malignant tissues in vitro

Cultivation of tumour cells in tissue culture date back almost to the beginning of tissue culture technique in general. The numerous results of this study illustrate the advantages of this technique as compared with the histological observations of sectioned material. In tissue culture the observations are made on the living material; cytological constituents, cell behaviour, cell movement, intercellular reactions and reactions to changes of environment can be studied. Moreover, the understanding and interpretation of fixed material is enhanced.

The method of cultivation of tissues in vitro was originated by Harrison (1907) who studied the outgrowth of nerve fibers and cultivated fragments of tissue from frog embryos in lymph obtained from the dorsal lymph sacs of the frog. Burrows (1911) perfected Harrison's technique for cultivation of tissues of warm blooded animals in homologous plasma. Carrel and Burrows (1911a) have reported growth of many adult tissues and glands of warm blooded animals.

Carrel and Burrows (1911b) were the first to succeed in cultivating rat and dog carcinoma and attempted, unsuccessfully, to obtain growth of carcinoma of human mammary glands. L'Ambert and Hanes (1911) cultivated mouse carcinoma cells. In both cases no difference was found between malignant and normal cells in vitro.

More than a decade passed before cultivation of pure strains of tumour was symstematically attempted. In 1924 Erdmann, working with Flexner-Jobling rat carcinoma in tissue culture, found that only cultures containing both tumour epithelial and stroma cells caused malignancy upon inoculation into animals. However, Fischer and Laser (1927) studying a similar strain, obtained tumours with inoculation of tumour cells free from stroma cells. Erdmann (1930)

and Fischer (1930) concluded that carcinoma cells grow as typical epithelial cells, but that tumour cells are not as uniform as the normal cells, and are more independent, since they sometimes grow out as single cells. Fischer also found that the carcinoma cells overrun the normal fibroblasts in tissue cultures - whereas the normal epithelial cells are themselves overrun by the normal fibroblasts.

Lewis and Strong (1934) cultured a number of spontaneous mouse mammary carcinoma cells and concluded that they grow like the normal epithelial cells with only the occasional one being independent. Moreover Lewis (1923) observed numerous isolated epithelial cells in cultures of amniotic ectoderm. Thus Fischer's conclusion of the nature of growth of carcinoma cells is not a general characteristic. In 1936 Gey and Gey reported the successful continuous culture of normal and tumour cells of man.

Cytology of malignant cells in vitro.

Virchow in 1851 was the first to arouse interest in cytopathology when he demonstrated the presence of inclusion of cellular nature in malignant cells. Since then many cytological differences between normal and malignant cells have been reported; most of these, such as variation of size and shape of the cell and nucleus, multiple nuclei and the presence of numerous mitotic figures are generally accepted. There has been much controversy over the possibility of recognizing the malignant tumours by examination of individual cells stained by special techniques for specific cytoplasmic and nuclear structures.

Lewis and Gey (1923) found that malignant cells of mouse carcinoma 180 could be identified in tissue culture. Since then the same has been found true of other tumours.

Fell and Andrews (1927) studied the cytology of Hensen rat sarcoma. They observed the cell constituents of the two types of cells occurring in this tumour. Their study included the mode of locomotion of cells, the formation of binucleate cells due to incomplete mitosis, the formation of multinucleated cells, and the cytological changes preceding cell death. As a result of these observations the authors stressed the advantages of tissue culture for cytological studies in general.

McCarty (1923, 1929) supported the contention that malignant cells can be identified. He focused attention on the nucleolus-nucleus ratio which he asserts is specific for normal and malignant cells. Other competent pathologists, including McCallum, claim that it is not always possible with "means now at our command" to distinguish a malignant cell from a benign tumour cell or even from a normal homologue. Huberg in Germany and Dudgeon in England are in agreement with McCarty's thesis that "cancer cell as an entity can be recognized in majority of cases by those who are trained in study of fresh tissues".

Dudgeon and Barret (1934) drew attention to the marked phagocytic properties of malignant epithelial cells, which are not found in the normal. In 1930, Horning and Richardson described another type of cytoplasmic inclusion consisting of extruded nuclear chromatin. In 1931, Levine, on the basis of his own observations and those of other workers, concluded that all tumours have variations in chromosome number within a lesser or greater range around the normal diploid number.

From the examination of thin films prepared from fresh specimens Fidler (1935) concluded that a malignant cell can be recognized in

suitable preparation by its morphology alone. His diagnosis was based mainly on the appearance of the nucleus, the irregularity of its position within cytoplasm - (usually eccentric, and larger and more variable in size). Nuclear chromatim is darkly-stained and more granular than the delicate structure of the normal prototype. The nucleolus is always very large, single or multiple; irregular in shape, and sometimes surrounded by a clear halo.

Other tissue culture experiments confirmed the fact that carcinoma retained its cytological characteristics and its malignancy after successive cultivation in vitro. Some workers maintain that the mode of colony formation and of proliferation of normal and malignant cells from the same type of tissue are remarkably similar. Incorporating his own observations with those of others, W. H. Lewis (1932, 1935) offered the following cytological criteria for differentiating tumour cells from their normal prototype in tissue culture; larger size of cell; more granular and denser cytoplasm; larger nucleus; more granular nucleoplasm; greater variation of chromosome number.

The causes may vary with different types of cancer and should be investigated for each type by a specific method. But, all investigators agree that the agent, whatever it is, releases the cells when malignancy is established from the regulatory complexes that govern growth and development of normal cells.

<u>Tumour production in vitro</u>.

There have been numerous attempts to produce malignancy by direct action of carcinogens. We shall mention only those attempts which have been made in vitro. As early as 1926, Fischer and in 1928 Laser reported

cancerization of embryonic tissues by using tar, arsenic acid, and x-rays. In 1935 des Ligneris claimed that he had produced transplantable tumours from mesoblastic cells of the chick by treating them in vitro for a month with lecithin emulsion of 1:2:5:6-dibenzanthracene. Only one of the six cultures which were injected into chickens produced a tumour at the site of injection. The experiment is not convincing since the possibility of development of spontaneous tumours has not been eliminated.

More recently Earle and Voegtlin (1940) in a series of papers have reported that prolonged treatment of normal tissue cultures with methylcholanthrene brought about an alteration in the cells. The production of transplantable malignancy from cultures of normal cells has not been demonstrated yet.

Frog Tumour in vitro.

Lucke (1934) reviewed seventeen reported cases of spontaneous tumours in frogs: of these 5 cases were adenomata, 4 adenocarcinomata of the skin, 2 hypernephromas of the kidney, 2 sarcomata of the extremities, 1 carcinoma of the ovary, 3 tumours involving intestine, mesentery and pancreas.

Murray (1908) studied a case of kidney tumour reported by Smallwood in 1905 which he determined had its origin in the adrenal. In 1932 Downs reported an epithelial tumour of the intestine of a frog. Lucke (1934a) who examined the sections of the two tumours reported by Smallwood and Downs, concluded that both growths had their origin in the kidney and were similar to the adenocarcinoma which he had systematically studied. In a series of subsequent papers he reported the geographical distribution of this tumour, the manner of metastases, and numerous homoplastic and hetereplastic transplantation experiments.

III. MATERIAL AND METHODS

1. Materials

Fragments of spontaneous tumour were obtained from Vermont and Eastern Canada frogs, Rana pipiens, and normal kidney from the same lot of animals. The frogs were kept in tanks at about 4°C and the material was taken over a period of four months beginning with November 1941. Embryonic kidney was obtained from 20 mm. tadpoles kept at 15°C. All living material was removed under strictly aseptic conditions in the sterile-air tunnel.

Chicken plasma was obtained aseptically from subclavian vein of young roosters; dog plasma from young dogs, through brachial vein; the frog plasma, by the heart puncture.

Chick embryo extract was prepared weekly from 8 to 10 day old chick embryo. The pulp was diluted with Tyrode Solution, which was allowed to stand for 30 minutes to 1 hour before centrifuging. The supernatant fluid was pipetted off. Frog spleen extract was prepared by the same method as the chick embryo extract from the spleen of healthy, normal frogs. Tadpole extract was prepared from tadpoles reared under aseptic conditions at room temperature. The extract was prepared as soon as the tadpoles hatched.

Feeding solution used throughout experiments was an artificial nutrient, prepared according to Baker's (1935) formula, but with salt concentrations modified for amphibian cultures. Fresh amphibian Ringer and Tyrode solutions were prepared frequently and their pH was always checked before using.

All glassware and instruments were sterilized in an oven at 160°C

for 1 hour. The solutions were autoclaved at 17 lbs. pressure for 1 hour. The plasma was obtained aseptically and checked for bacterial contamination. The feeding solution was sterilized by filtering through a Chamberlain - Pasteur candle.

2. Methods

Essentially the classical tissue culture technique was employed in the experiments. Some modifications were introduced as required by the specific needs of the investigation.

Carrel flask method and roller tubes and roller bottles (modified Lewis and Gey technique) were used for maintenance of cultures over long periods. In all cultures fragments of tissue usually 1 to 2 mm^2 in area were planted in a thin layer of plasma which was coagulated by the addition of chick embryo extract;the clotted layer was bathed by nutrient solution, and the bottles and tubes were rotated at a speed of about 16 revolutions per hour. Carrel flasks were shaken by an adaptor to the rolling apparatus, with the same speed and with a 30° amplitude or allowed to remain stationary.

For cytological studies of cultures the "lying drop" or "flying coverslip" method of Maximov was employed. (Small round coverslip attached by fluid to a larger square one and inverted over a depression slide). Tumour fragments were implanted into the drop of coagulating plasma; normal kidney fragments were usually planted over the surface of clotted plasma to facilitate the growth of epithelium. A drop of nutrient medium was added to the solid medium in all coverslip cultures.

The solid medium in the cultures usually consisted of equal parts of heparinized chicken and frog plasma which was solidified by addition of diluted chick embryonic extract. The nutrient solutions were modifications

of those used by Lewis, Vogelaar and Baker for mammalian tissues. The salt components were replaced by those of amphibian Ringer and the medium was hypotonized to adjust for the osmotic pressure of frog plasma. The homologous serum was frequently omitted. The treatment of cultures, and the frequency of renewal of media varied for the tumour and normal cultures as well as for the specific temperature at which they were kept. Generally, the supernatant fluid was renewed every 3 to 4 days and the plasma clot was patched were necessary in the tubes and Carrel flasks.

The "lying coverslip" cultures were transplanted every 3 to 5 days. The "flying coverslip" cultures were bathed in Tyrode every 3 to 4 days and remounted on fresh coverslips after an addition of fresh medium. The Carrel cultures were transplanted every 5 to 7 days (less frequently for normal); the roller tubes, every 2 weeks. The pH of the cultures was adjusted by passing a stream of air or of CO2 though them. The cultures were kept in an incubator at 25°C ± 0.2 or at room temperature (23° to 25°C) Other temperatures used to test the temperature tolerence of various cultures will be discussed later.

Growth was recorded by means of the projectorscope or camera lucida drawings. The area was measured with a planimeter. Microscopic observations of cytological details with high magnification were made directly on vitally stained coverslip or Carrel flask cultures. Routine camera lucida drawings were made; representative cultures were photographed; others fixed in "Susa" or neutral formalin, and stained <u>in toto</u> with hematoxylin - eosin and other stains, or fixed and stained with aceto-carmine.

Table I gives type of medium used, relative proportions of constituent parts of clot and nutrient fluid medium, the type of tissue, intensity of the growth obtained and maximum number of days that the tissue was maintained in a particular medium.

3. Experimental Media

Chick embryo extract, frog spleen extract, and tadpole extract were added in various combination with other media to some cultures. Type I medium (Table I) was used as control in all cases for both normal and malignant tissues.

The pH of the medium was adjusted by the addition of sodium bicarbonate to the Tyrode or to the feeding solution which contained 0.05% of phenol red and was determined colorimetrically. For extreme pH adjustment, phosphate buffer solutions were used.

The temperature effect was studied by maintaining cultures at the following temperatures: 38°C ± 1°, 15°C ± 2°, and 3°C ± 1.0°.

Indian Cobra venom in dry crystals form was first diluted with distilled water and added to the cultures with Tyrode solution as a nutrient. The solid medium used was Type I (Table I). The final concentration of cobra venom was 1:50,000.

Potassium cyanide was added to Type I medium to make a final dilution of $\frac{1}{100}$ and $\frac{1}{1000}$ molar. Iodoacetic acid was added in similar concentration. In all cultures the original medium was replaced after the periodic washing with Tyrode solution and subsequent "patching" of the clot.

TABLE I

Composition of Media, Intensity of Growths and Duration of Cultivation

TYPE OF MEDIUM	Composition of Media: Solid Medium (Parts in 20) #)					Nutrient Medium (Parts in 10) #)						Tissue and Intensity of Growths ##)			Maximum Number of Days in Medium	
	Chicken Plasma	Frog Plasma	Dog Plasma	Chick Embryo Extract 50%	Tyrode Solution	Chick Embryo Extract 50%	Spleen Extract 35%	Tadpole Extract 50%	Frog Serum	Tyrode Solution	Synthetic Nutrient Solution	Normal Kidney	Tumour Tadpole Kidney		Normal Kidney	Tumour Tissue
I	5	5		2	8							3	3	3	50	35
II		5	5	2	8	2	-	-	2	-	6	2	1	0	23	10
III	9			2	9							2+	3	2	18	26
IV	5	5		2	8	-	6	-	-	4	-	2	1	-		
V	5	5		2	8	2	-	-	-	2	6	2	3-	-	34	49
VI	5	5		2	8	4	-	-	-	6	-	1	2	-	20	20
VII	5	5		2	8	-	-	4	-	6	-	2	?	3	14	-

#) Amount of Media in Cultures

Type of Culture	Solid Medium (cc.)	Nutrient Medium (cc.)
Carrel Flasks	1.2	0.7
Roller Tubes	0.4	1.0
Roller Bottles	0.5	1.0
Depression Slides	0.1	0.05

##) Numbers indicate intensity of growth (area - increase) in terms of that of Type I Medium.

3 = Good
2 = Fair
1 = Poor
0 = No growth

IV. OBSERVATIONS

Manner of Growth of Normal Kidney and Tumour Cultures

Most of the observations of normal kidney growth were made with cultures that had been transplanted at least once after the original excision because, unlike the tumour cultures, the growth of normal epithelium begins only after a latent period of several days. Since we have not succeeded in obtaining pure cultures of normal kidney epithelium, other cell types taking part in the growth of normal kidney explants will also be considered.

(a) The Normal Frog Kidney

Lymphocytes and macrophages begin migrating out of the fragment soon after explantation. After a day or two fibroblasts are seen in the medium as well as at various points of the margin, (Plate I, Fig.10). A day later endothelial cells migrate out sometimes forming leaf-like clusters atcertain points of the culture. Generally around the fourth day the epithelial proliferation begins. These sheets of cells usually grow over the fibroblasts which by that time completely encircle the fragment, (Plate I, Fig.11). Occasionally sheets of epithelial growth were seen over the surface of the clotted plasma, (Plate I, Fig.12). We have not obtained pure epithelial cultures regardless of the number of transplants, though sheets composed of pure epithelial cells were observed in about fifty percent of the cultures. These cells were fairly uniform in size sometimes having a pavement arrangement. The kidney tubules sometimes showed growth of cells but the tubular arrangement was always lost, the cells spreading into an undefferentiated membrane.

(b) Malignant Tissue

Soon after explantation budlike protrusions appeared at various points of the margin of the fragment. The margin as well as the budlike structures becomes quite translucent. The hemispheric buds are composed of radially arranged long pyramidal cells with the broad apex at the periphery. With the advance of growth the buds elongate appearing like meristematic root tips. These cylindrical structures without lumina may retain their straight cylindrical form, but more frequently begin to twist and bulge at various points because of the uneven growth rate, (Plate II, Fig.14). The margin of the fragment becomes uneven; the bulging cylinders predominate, though many of the budlike structures expand in diameter rather than in length, (Plate II, Fig.18). When these cylindrical structures contact the glass their sharp outline is lost and the sliding of cells onto the glass surface begins. The cells flatten out as they adhere to the glass, and expand into a unicellular fan-shaped membrane showing a pavemented epithelial arrangement, (Plate II, Fig.16). Cell membrane growth was seen in some cultures on the second day after explantation, but in all cultures it was most conspicuous on the third or fourth day when the fan-shaped growths fuse with each other framing the whole explant with a broad lace-like band of tumour cells, (Plate II, Fig.15 and 16.

The membrane consisted entirely of tumour cells adhering to each other, The stroma cells and macrophages that were present during the first few days became less conspicuous. The migration of individual tumour cells from the margin of the growth is seen from the very beginning of membrane formation, and is more marked with the advance of

TABLE II

Mean Relative Area-Increase of Normal and Malignant Cultures in Type I Medium

Type of Tissue	Number of Cultures	Mean Relative Area-Increase (± mean standard error) 24 hours	48 hours	72 hours	96 hours	120 hours	144 hours
Normal	47	0.118 ± 0.07*	0.265 ± 0.03	0.392 ± 0.05	0.647 ± 0.07	1.021 ± 0.11	1.272 ± 0.18
Tumour	40	0.141 ± 0.02	0.38 ± 0.07	0.668 ± 0.09	1.693 ± 0.25	2.636 ± 0.36	3.889 ± 0.66

* Normal cultures show area-increase only after a latent period of 3 to 4 days. The hours are from the time the increase becomes apparent.

TABLE III

Mean Daily Increment of Area of Normal and Malignant Cultures in Type I Medium

Type of Tissue	Number of Cultures	Mean Relative Daily Increment 24 hours	48 hours	72 hours	96 hours	120 hours	144 hours
Normal	47	0.118	0.1305	0.104	0.164	0.206	0.1787
Tumour	40	0.141	0.220	0.211	0.620	0.264	0.283

liquifaction of the coagulum.

Extent of Growth

The comparative relative area-increase of normal and tumour cultures was determined by daily measurements of the areas of each culture. All cultures in this experiment were maintained in Carrel flasks or on "flying coverslips" placed over depression slides in identical media (Table I, Type 1 medium) and at the same temperature (25°C).

(a) Mean Total Area-increase of Tumour and Normal Kidney Cultures

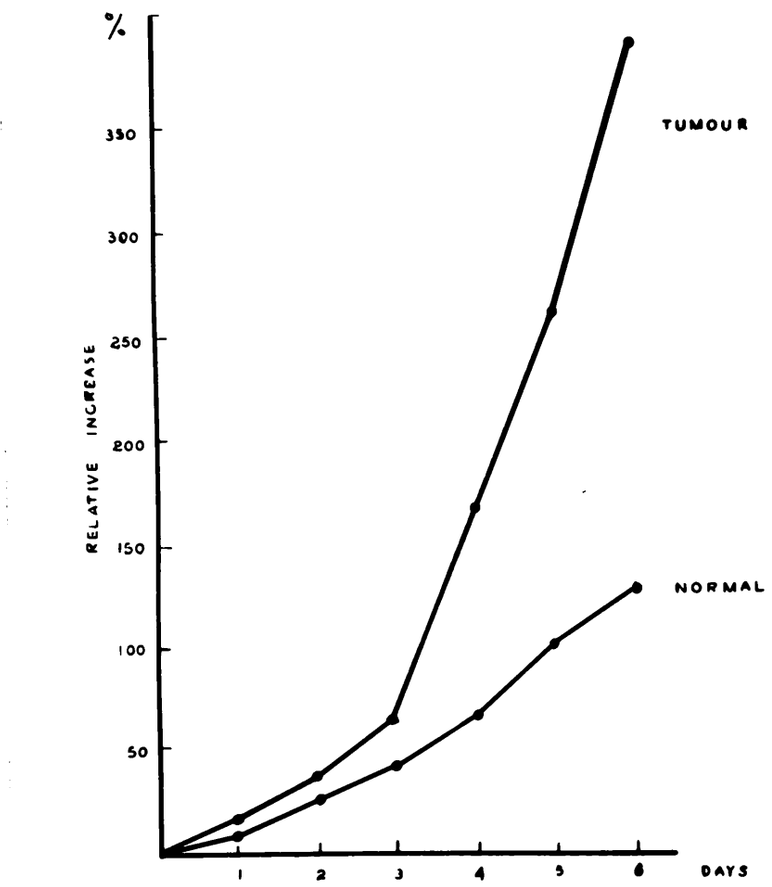
The relative increase of each culture was determined by expressing the difference between the new area and the original area in terms of percentage of the original area. The formula is:

$$\% \text{ relative increase} = \frac{An - Ao}{Ao}$$

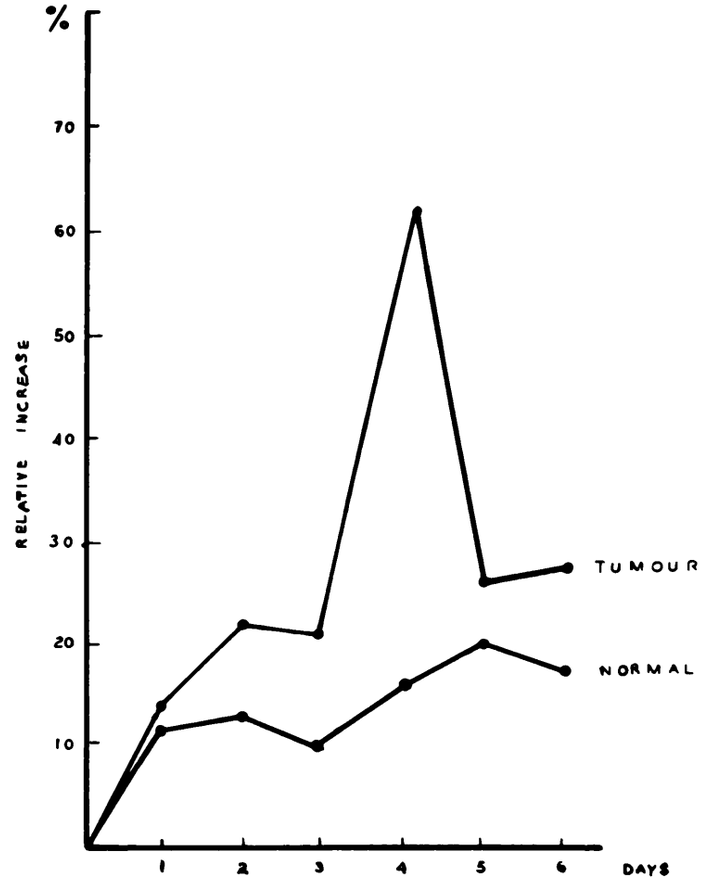
where An is the area of the fragment at the end of the particular period and Ao is the area at the beginning of the period. Table II gives the summary of these calculations; statistically the difference between the area-increase of normal and malignant cultures is not significant for the first and second days. However, the relatively small number of cultures and the considerable variations within each group may account for this fact. Text fig. 1-A represents graphically the mean total relative increase of normal and tumour cultures from the figures summarized in Table II.

(b) Mean Daily Increment

The analysis of the daily changes of the areas of the cultures



A. MEAN TOTAL AREA INCREASE



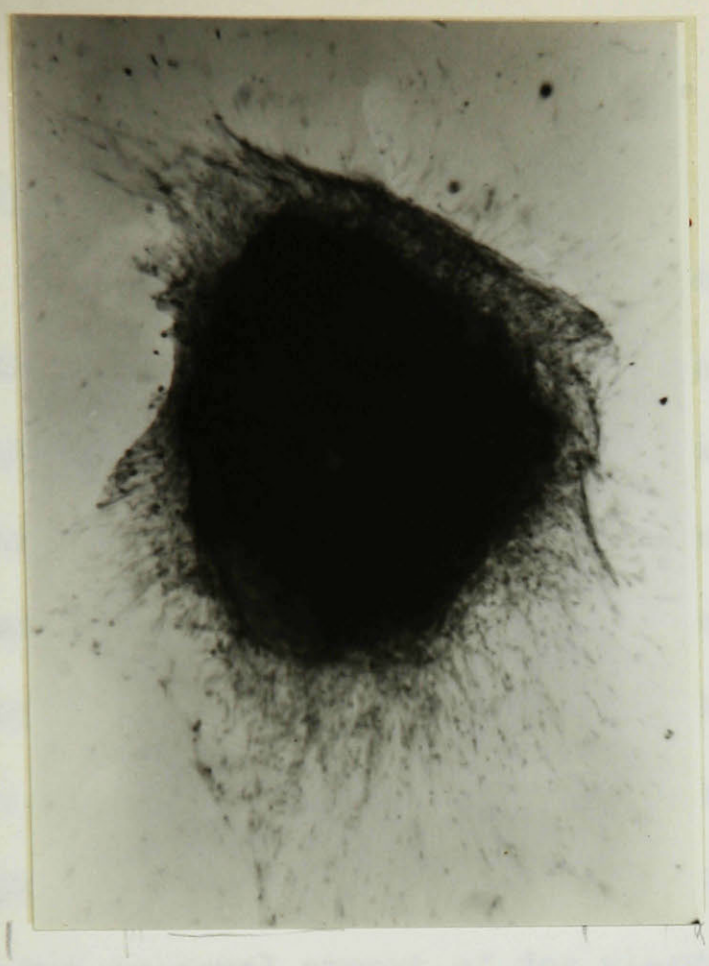
B. MEAN DAILY INCREMENT

Text Fig. 1. - A. Mean total area-increase of normal and malignant cultures in Type I medium. (For normal cultures: time in days after the first appearance of growth. See text).
B. Mean daily increment of normal and tumour cultures in Type I medium.

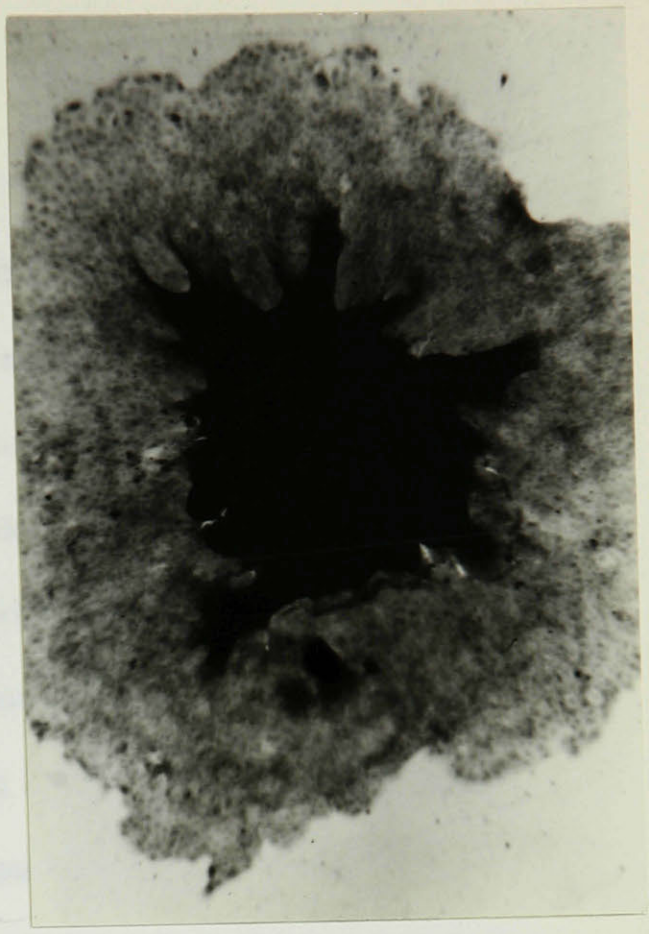
reveal the changes in metabolic activity or manner of growth. Text fig.1-B represents the respective means of daily increment for the tumour and normal cultures by expressing the absolute increase of the area for one day in percentage of the area of the preceding day. The summary of calculation is in Table III. The cultures of tumour explants were measured immediately after transplantation and daily thereafter. The normal cultures were measured soon after transplantation; but since, due to a latent period of 3 to 4 days no growth was evident, the day on which an increase of area appeared was considered the first for the calculations. The maximum daily increment of the tumour cultures is due to the spreading of cells on the glass surface which is most conspicuous during the fourth day when liquifaction of the medium occurs. The difference in relative increases of area, both total and daily, is more striking when these are observed in two typical normal and malignant cultures (Text fig.2, 3 and 4).

Effect of Media on Growth of Normal and Tumour Tissues

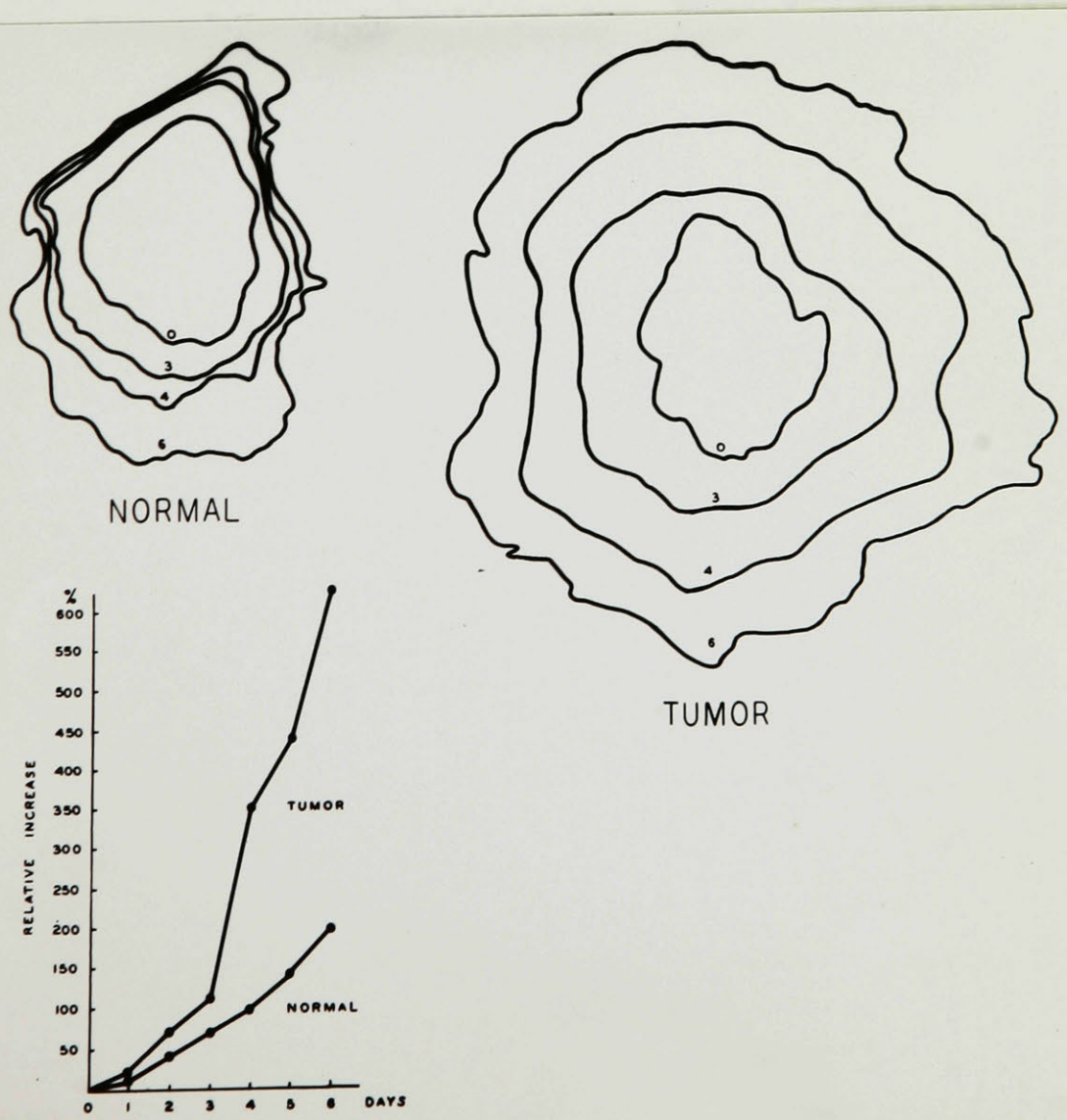
The tissue cultures used in our experiments were composed of two phases: one, a solid medium made up from different plasmas in various proportions and coagulated by the addition of chick embryo extract; the other, a supernatant fluid medium consisting of different extracts and nutrient solutions in various proportions. The solutions to be tested were added to the fluid medium while the components of the coagulum remained constant, and in that manner the effect of a particular substance on the manner of growth or the metabolic activity of the culture was observed.



Text Fig. 2. - Normal Kidney culture in Type I medium showing 6-day area increase. (The lower margin of epithelial growth became folded in fixation.) (x 45)



Text Fig. 3. - Frog tumour culture in Type I medium showing 6 day area increase. Fig. 3 and 4: Neutral formalin, hematoxylin-eosin. (x 45)



Text Fig. 4. - Diagrams showing the increase in surface area of the two cultures shown in Text fig. 3 and 4. The graph shows the relative area increase of the two cultures.

(a)Plasma and Serum

Various combinations of solid media were tried in order to obtain a medium producing optimum growth in normal as well as in tumour cultures. The greatest relative increase in area for both types of cultures was obtained in media containing 5 parts of chicken plasma, 5 parts of frog plasma, 2 parts of chick embryo extract (50%), and 8 parts of Tyrode solution (Table I, Medium Type 1). The intensity of area-increase for other media was determined by comparing with the mean relative increase in Type I Medium. When the proportions of substances were kept the same but an equal amount of dog plasma was substituted for chicken plasma (TableI, Type II) relatively better growth was observed in normal than in malignant cultures. Tumour cultures grew slightly better than normal ones when frog plasma was omitted from Type I medium (Table I, Type III). The supernatant nutrient fluid was kept constant in these three series of tests; all were incubated at 25°C.

Keeping the solid components constant and using the Type I medium the effect of various combinations of nutrient fluids were observed. Largest area-increase resulted in both tumour and normal cultures with a medium containing 2 parts of frog serum, 2 parts of chick embryo extract and 6 parts of Baker's modified feeding solution (Type I). When 6 parts of 35% spleen extract were combined with 4 parts of Tyrode solution (Type IV) very slight area-increase of tumour and a considerably better growth of normal kidney resulted (Text fig.5). Omission of frog serum (Type V) produced a better growth in the tumour cultures where it was almost as extensive as in Type I medium. Similarly better results were obtained with tumour cultures than with normal when the nutrient solution

was omitted (Type VI); the normal cultures increased only slightly.

The effect of tadpole extract (Type VII) is not conclusive. Bacteria-free tadpoles are difficult to obtain since there is no efficient method of sterilizing either the frog eggs or the sperms. Some contamination was always present in spite of the fact that all the operations were carried on in sterile-air tunnel. Somewhat better results were observed in normal kidney cultures but the constancy of the environment is questionable, and new factors may have been introduced.

(b) Snake Venom

In 1936 des Ligneris and Grasset reviewed a number of experimental studies on the treatment of human cancer with snake venom. In some cases there was a relief of pain; in others no such effect was obtained. In several cases snake venom produced astimulating effect on the whole organism. Occasionally, there was an arrest of growth of tumour tissue but the effect was uncertain and not permanent.

Chopra et al (1936) have demonstrated the growth promoting quality of cobra venom in low dilution on chick embryo tissue in vitro. We have tried Indian cobra venom in low dilution (1:50,000) on cultures of normal and tumour tissues. In neither was the growth promoting effect observed. The tumour growth was usually smaller than that of the normal; some cultures showed no growth at all. The best culture showed a marked vacuolization of cells and abnormal swelling of cytoplasma, the fact that suggested that the addition of cobra venom reduces the tonicity of the medium, the metabolism or the respiratory processes. The results have shown that cobra venom has a considerably greater inhibiting effect on tumour cultures. Plate III, Fig.18, 19 and 20 show the appearance of

cells in venom cultures. The vacuoles are conspicuous in all cells and the margin of cytoplasm is sharply defined. The usually ruffled cytoplasm of tumour cells was not observed.

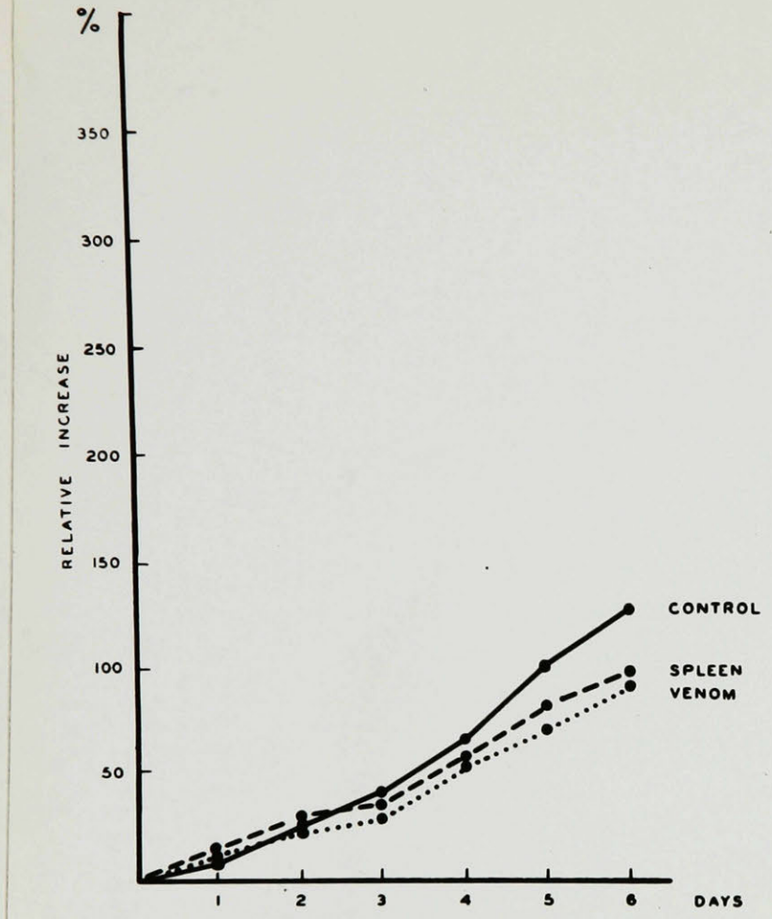
Respiratory Poisons

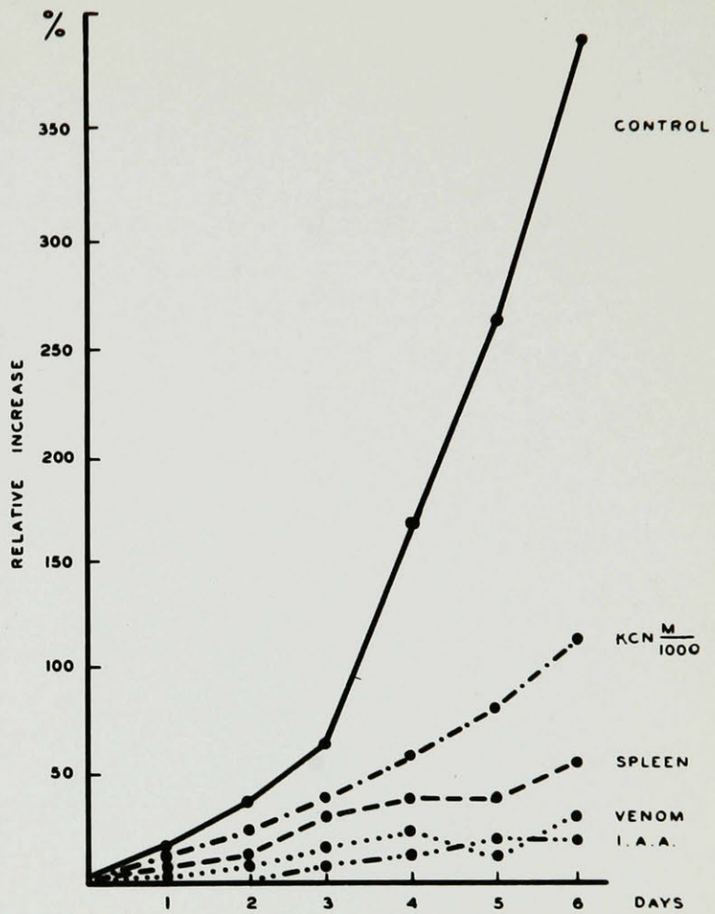
Dilutions of KCN and Iodoacetic acid were added to the cultures in Type I medium. The pH of the cultures with $\frac{1}{100}$ molar KCN had to be readjusted. The concentrations of $\frac{1}{100}$ and $\frac{1}{1000}$ molar KCN inhibited the growth of the normal cultures completely; no measurable increase being noted over many days. In the latter concentration of KCN normal tissue appeared to be in a better condition.

Iodoacetic acid rendered the medium too acid. The pH was adjusted by addition of small amount of 1N. KOH. Most of the tumour cultures showed no growth at all; others, showed only a slight increase in area. The normal kidney cultures were similarly affected, but whether the effect was due to presence of Iodoacetic acid or to some other factor is not known. The experiment designed to show the effect of Iodoacetic acid on normal kidney is, therefore, not conclusive. Text fig.5 shows the mean relative area-increases in media containing KCN, Iodoacetic acid, venom, and spleen extract. The general appearance of tumour fragments and of tumour cell membrane are shown in Figures 21 and 22, (Plate III,).

The Effect of pH

All the physiological solutions contained sodium bicarbonate; the latter being autoclaved separately and added to the solutions immediately before use to avoid precipitation. The pH was checked. The adjustment of pH was effected by passing a stream of air or CO_2 through





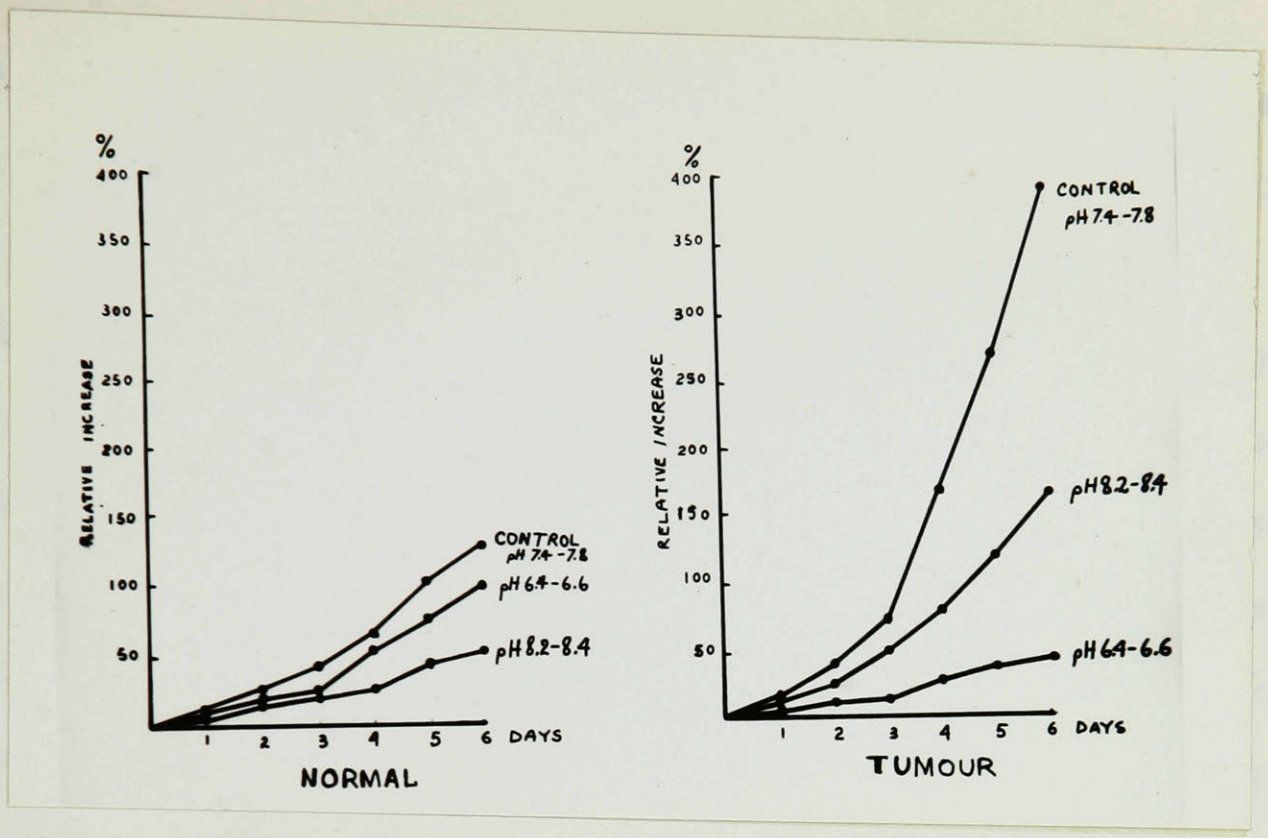
Text Fig. 5. - Mean relative area-increase of normal and tumour cultures in media containing spleen extract, snake venom, KCN, and Iodoacetic acid (I.A.A.) Normal kidney cultures showed no visible increase of surface area in media containing KCN and Iodoacetic acid.

the media. The effect of pH on normal and tumour tissue was studied over a range from pH 6.0 to pH 8.5. Extremes of pH were adjusted by the addition of phosphate buffers.

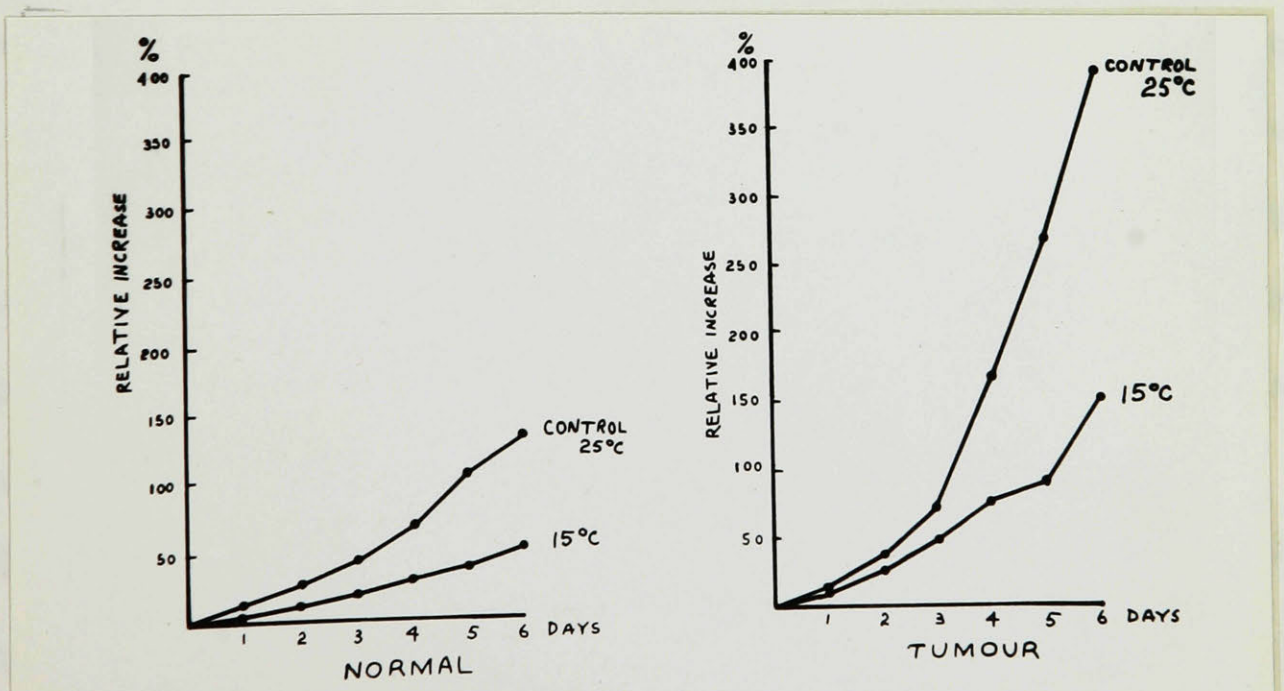
In solutions more alkaline than pH 8.2 - 8.4 the tumour tissues tended to adhere to one another and differed markedly from controls (pH 7.4 - 7.8) in total growth. The marginal tumour cells tended to disintengrate more rapidly in a pH lower than 6.8. Presumably the effect was primarily on the cell membrane. No similar effect was observed with normal tissue which remained viable even in media as acid as pH6.0 or as alkaline as pH 8.8 though no growth was observed in the latter medium. At pH 6.0 many of the normal cells showed considerable injury. The vacuolation was particularly marked in both normal and malignant cultures; the tumour cells being more sensitive to acid media. Other changes of cytological nature were not noted. The most striking effect of pH variation is on the intensity of growth. The comparative relative area-increases of normal and malignant cultures are shown graphically in Text fig.6. It is interesting to note that the normal kidney cultures showed a relatively greater area-increase in acid media. The effect of alkaline media was different; the increase of area of tumour cultures was greater than that of normal.

Effect of Temperature

Considerable variations are inherent in the range of temperature of the normal environment of the frog. Since the frog has no temperature regulating system, it readily assumes the temperature of its surroundings. The influence of temperature on the rate of normal growth and development has long been established and it is more direct in its expression in the



Text Fig. 6. - Relative area-increase of normal and tumour cultures in different pH.



Text Fig. 6-A. - Relative area-increase of normal and malignant cultures in different temperatures.

frog than in warm blooded animals because of the foregoing factors.

A series of experiments designed to test the temperature effect on normal and malignant cultures was carried out. The cultures were either freshly made from frogs kept at 22° - 25°C for at least two days prior to the excision of fragments or from transplants of cultures maintained in Type I medium at 25°C. No difference in the effect was observed between the two types of cultures. The cultures - Carrel flasks and depression slides all containing Type I medium - were cultivated at different temperatures and compared with a number of controls kept in an incubator at 25°C ± 0.2.

In the first group of experiments the cultures were transferred to 3°C immediately after preparation (Group A) or after 72 hours of incubation at 25°C (Group B). The Group A, normal kidney cultures, showed no growth at all though many cells were found in the medium in the neighbourhood of the fragments (Plate IV, fig.25). Tumour cultures of Group A showed some suggestion of budding. No measurable growth was observed for a period of 30 days in the case of normal and for 10 days in tumour cultures. Both cultures resumed their growth when brought to 25°C: the normal kidney cultures attained a typical 48 hour-growth in 96 hours; the tumour cultures showed a typical area increase.

In the second and third groups of experiments a similar procedure was followed. The normal and malignant cultures were exposed to temperatures of 15°C and 38°C. Both normal and malignant cultures showed retardation of growth as compared with the controls (25°C). None of

TABLE IV.

Mean Relative Area-Increases in Normal and Malignant Cultures in the Experimental Media

Number of Experiment	Type of Medium	NORMAL: Number of Cultures	NORMAL: Mean Relative Area-Increase 24 Hours	48 Hours	72 Hours	96 Hours	120 Hours	144 Hours	TUMOUR: Number of Cultures	TUMOUR: Mean Relative Area-Increase 24 Hours	48 Hours	72 Hours	96 Hours	120 Hours	144 Hours
I	Spleen	27	0.130	0.280	0.360	0.595	0.815	1.020	14	0.063	0.124	0.307	0.327	0.392	0.563
II	Venom	18	0.125	0.240	0.320	0.535	0.720	0.960	16	0.028	0.092	0.164	0.252	0.131	0.333
III	pH8.2-8.4	13	0.035	0.120	0.180	0.280	0.420	0.510	12	0.098	0.245	0.475	0.790	1.130	1.655
IV	pH6.4-6.6	11	0.080	0.198	0.315	0.565	0.715	0.940	14	0.035	0.075	0.123	0.270	0.330	0.365
V	15°C	18	0.045	0.115	0.173	0.245	0.350	0.470	11	0.093	0.270	0.475	0.715	0.840	1.385
VI	$\frac{M}{1000}$ KCN	14	-	-	-	-	-	-	19	0.130	0.250	0.400	0.595	0.810	1.130
VII	$\frac{M}{1000}$ IAA *	?	-	-	-	-	-	-	8	-	-	0.090	0.137	0.215	0.215
VIII	Control **	47	.118	0.265	0.392	0.647	1.021	1.272	40	0.141	0.385	0.668	1.693	2.636	3.884

* Iodoacetic acid

** Controls: Type I medium, pH 7.4 - 7.8, 25°C

the cultures showed any increase of area at 38°C. The nuclei appeared moribund after 12 hours exposure to that temperature; all died after 48 hours. Text fig.6-A showsthe results of the temperature experiments. This is a graphical representation of figures given in Table IV; other calculations obtained from the experiments designed to test the effect of various experimental media described above are also included in this table.

Cytology of Normal and Malignant Cells

The comparative study of cells included the normal kidney epithelial cells, tumour cells and tadpole mesothelial cells. Other cells abundantly present in all cultures are not considered here but will be referred to.

Changes in constituents of the medium has a marked influence on the cell shape. Without a support tissue cells round up and disintegrate. The amount of fibrin in the coagulated plasma determines what form a specific cell would assume in tissue culture. Even in identical media different cells may cause certain changes to take place by their infiltrating activities or with the help of their metabolic products; these in turn may bring about a subsequent alteration in cell form.

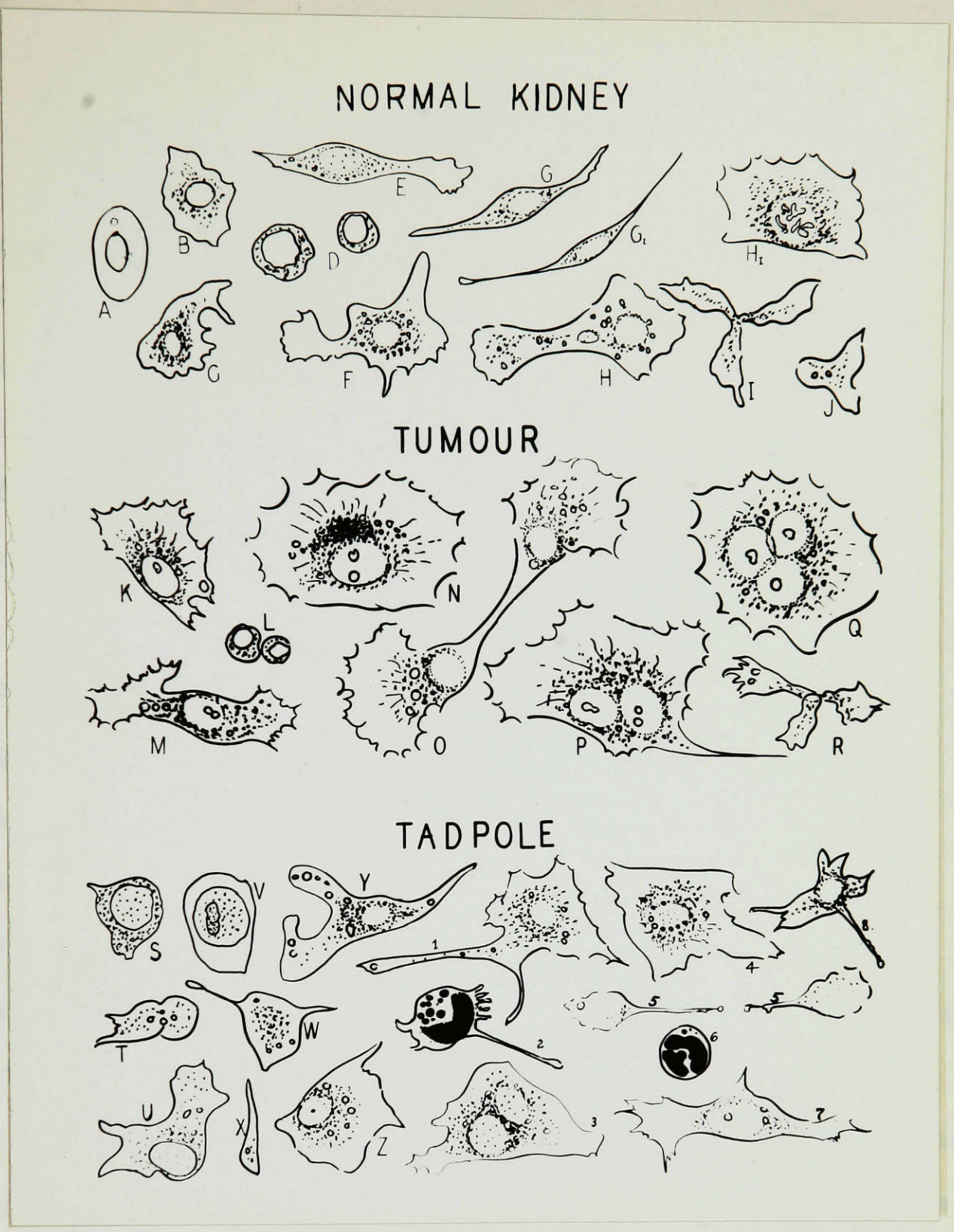
Malignant cells are much larger in vivo than their homologue, the normal kidney cell. The difference in size is accentuated in vitro though there is a considerable variation in the size of tumour cells. The cytoplasm of tumour cells is denser and more granular, but the band which surrounds the dense granular central region is invariably wider in tumour cells when the cells flatten out on the glass surface (Plate V, Fig.29 and 35). The margin of isolated tumour cells is difficult to distinguish but becomes visible with careful focusing and adjustment of light. Isolated normal cells have a more definite outline; the tadpole cells have the sharpest outline (Plate V, Fig.30; Plate IV, Fig.26 and 27).

The marginal activity of cytoplasm is more conspicuous in tumour cells. The ruffled pseudopoda are particularly active in cells that are in locomotion (Plate V, Fig.28). Such tumour cells were seen to entrap portions of

the medium and incorporated them as discrete globules into their cytoplasm (pinocytosis). Although the cytoplasm of tumour cells appears more granular, the Neutral Red vacuoles are less conspicuously stained in these than they are in the normal kidney epithelial cell. The staining appeared less pronounced, the vacuoles being yellowish in colour, so that it was difficult to distinguish them from the fat globules and other inclusions. In the living as well as in the fixed tumour cultures the fat vacuoles are very large.

The mitochondria are filamentous and longer than the more compact structures of the normal kidney epithelial cells. Since these structures, due to their length, extend beyond the central dense portion of the granular material which is surrounded by a chain of fat droplets, they stand out more conspicuously in the fairly homogenuous clear bend of cytoplasm. The estimation of quantitative differences of mitochondria present in tumour and normal cells was not attempted. Under favorable conditions and with proper lighting the centrosphere may be distinguished in the dense central granular region to one side of the nucleus in tumour cells. This structure was not seen in normal kidney cells.

The nuclei of tumour cells are generally larger than normal; there is considerable variation in size, however, and some overlapping with normal was noted. The nucleo-plasm is more granular in living tumour cells than in normal ones. The malignant cells invariably have one and sometimes two nucleoli. The nucleoli were occasionally in tadpole cells but were rarely seen in normal kidney cells (see Plate V, Fig.31, 32 and 35). Text fig.7 shows camera lucida drawings of different cell types found in normal, tumour and tadpole kidney cultures.



Text Fig. 7.- Camera lucida drawings of different cell types found in the normal frog kidney, the malignant kidney tumour and the tadpole kidney cultures. Approximate magnification x 500.

Normal kidney; A - Frog red blood cell; B - Epithelial cell (24hours); C,F - Epithelial cells (96 hours); D - Lymphocytes; E - Endothelial cell; F,H - Epithelial cells in locomotion; I - Endothelial cells in cobra venom; J - Lymphocytes (120 hours); G, G_1 - Fibroblasts. G_1, H,H_1 - 48 hours at 25°C after preliminary exposure to 3°C for 30 days.

Tumour: K,N - Tumour epithelial cells (24 and 120 hours); L - Lymphocytes; M - Tumour cell in locomotion; O - Tumour cell in mitosis (telophase); P - same cell as in O, 8 hours later; Q - Trinucleate tumour cell; R - Stroma cells.

Tadpole: S - Epithelial cell (24 hours); T - Macrophages; U,Y,Z,1,4,7,3 - Mesothelial cells in cultures from 48 to 280 hours; V - Unidentified cell; W,2,6 - Melanophores; X - Endothelial cell; 8 - Macrophage (?); 3 - Binucleate mesothelial cell.

(a) Mitosis in Tumours

The estimation of the rate of cell division in normal and malignant tissues was not attempted. Mitosis of normal kidney cell is difficult to observe because of the cellular substratum over which the epithelial cells proliferate. Tumour cultures are particularly favorable for observation of cell division because the cell membrane is unicellular in thickness and is in the immediate contact with the glass.

Preceding cell division a tumour cell becomes spherical by withdrawing most of its processes, one or two of which may sometimes persist. Apparently a change in surface tension enables the cell to detach itself from the glass. The cell in prophase becomes more spherical with a sharply defined outline. The time from the beginning of rounding up to metaphase is about 50 minutes to 1 hour for the tumour cells observed. The metaphase lasts about 20 minutes; anaphase, about 10 minutes. As the chromosomes proceed toward a pole, the cell elongates considerably and a constriction appears at the middle of the cell. The cleavage is rather rapid - the whole process being completed in about 9 minutes. During anaphase bulging processes are pushed out and withdrawn. The two daughter cells which are still connected by a hyaline strand of cytoplasm move apart slowly. The movement is a combination of flattening of the cells and simultaneous drifting. Within one hour the cells had flattened out completely, the nuclei became visible and were surrounded by mitochondria and cytoplasmic conclusions. The connection between the newly formed daughter cells persists as the cells move apart; it may become very thin, almost threadlike, and eventually

break. The broken ends appear clublike and are reabsorbed by their respective cells. Sometimes this connection persists. On one occasion it was seen to widen as the two cells approached and eventually merge into one binucleate cell (Text fig.7, P and O).

Whether all binucleate cells seen in the tumour cultures are formed by such a process is difficult to say, but mitosis of binucleate cells could give rise to a tetranucleate cells that were frequently seen in tumour cultures (Plate V, Fig.32). Binucleate cells were seen in tadpole cultures as well, (Text fig.7, 3). The formation of trinucleate cells has not been observed though such cells were seen in many tumour cultures (Plate V, Fig.31).

V. DISCUSSION AND CONCLUSION

Some preliminary remarks bearing on the general nature and behaviour of cultures in vitro should be made before interpreting the results obtained in the foregoing experiments.

When fragments of tissue are excised from the organ the separation from other cells and the injury of the marginal cells create an abnormal condition. This may be enhanced by the fact that the tissue is bathed for a considerable time in Ringer solution which does not constitute a normal environment for the cells. But if these conditions are kept constant for several tissues to be studied in vitro, it offers an opportunity for observations of reactions of these tissues to media which can be changed at will. Besides, a fragment in tissue culture should be considered as a primitive organ behaving as a whole.

Rate of growth may be influenced by many factors:

(a) The manner in which a fragment is cut out of the organism.

(b) The condition and size of the fragment.

(c) The time lapsing between excision and imbedding into the medium.

(d) The preparation and preservetion of media.

(e) The nature and quantity of substances used.

(f) The temperature of the incubator, and even small osmotic pressure difference.

To eliminate variation and sources of error considerable uniformity of the technique has to be observed. In our experiments all series of cultures where the controls showed atypical condition of growth were dis-

earded. The comparison of growth was made in the same series of cultures in which all other factors except the experimental ones were fairly uniform.

Manner of Growth

Explants of frog tumours taken at different times from different animals exhibit specific variations when grown in the same medium. Some have shown extensive growth of cylindrical structure which subsequently proliferated into a luxurious sheet-like growth either on surface of the coverslip or on the surface of the plasma. Others have shown some cylindrical outgrowths but very little sheet-like growth. Still others have shown no growth at all. Thus the growth depends upon the state of the tumour; that the growth fluctuates in vivo and in vitro has been observed by workers with other tumours. Frequently variations of growth were observed in fragments taken from different sites of a single tumour. This is closely correlated with the conditions prevailing in the tumour itself. Histological sections of several tumours have shown that certain parts were abundant with stroma tissue frequently obliterating the typical tubular arrangement. This fact confirms the observation of different growth rate in vitro: only the actively growing portion of tumours show extensive and continuous growth.

Apart from many others, two factors are particularly significant in the manner of growth in tissue cultures (in solid media) (a) the ability of the cells to infiltrate the plasma clot, and (b) the necessity of a supporting framework. Even in the presence of the medium which is rich in nutrient material and in substances stimulating proliferation,

the specific property of the tissue and the availability of a supporting framework will determine whether growth of normal tissue will occur, and the particular form that it will take. The normal kidney epithelium would grow soon after explantation by the resources of its residual growth energy if a suitable substratum could be provided. When imbedded in the plasma clot the epithelium begins proliferating only when a layer of fibroblasts has infiltrated the medium "using" it as a substratum; hence the latent period in the epithelial growth of normal kidney cultures.

Tumour fragments, as has been described above, show growth immediately after explantation. The growth takes the form of budding protrusions composed of columnar cells which elongate into tubule-like structures without lumina and assume the shape of irregular and twisted bulges due to unevenness of growth rate. This continues until a contact is established with the glass or, less frequently, with the surface of the clot.

The normal epithelium is seldom seen growing on the surface of glass because the liquifaction of the medium is very slow, while the substratum of fibroblasts is available before sufficient liquifaction takes place. Isolated normal epithelial cells round up in liquid medium. The cuboidal or columnar shape of cells is maintained in tissues (in vivo and in vitro) by the surface forces that act between homologous cells, by the particular affinity of molecular structures of cell membrane, by the cement substances that may be laid down between the cells, or through the action of all three factors which may be specific for each type of tissue.

The normal kidney epithelium, particularly that of the tubules,

maintains a rigid structure by means of intercellular substance. Other epithelial structures as frequently seen in many parts of the body, require a connective tissue framework for their support. In tumour cultures the formation of bud-like structures is fairly rapid. Since no framework is available as the medium is liquified, the mere force of gravity draws the cylindrical structures down; on contact with the glass its cells spread out since their affinity for the glass is apparently greater than for each other.

This lack of rigidity of tumour cultures is further confirmed by observations of the slide which was accidentally left with the coverslip side up ("hanging drop" rather than a "lying drop"). While other cultures showed considerable marginal growth after 72 hours, the growth was very scanty in this slide. But, since the culture looked healthy transplants were made from it. Upon examination the fragment showed a conspicuous mound-like appearance; presumably most of the growth being concentrated at the apex of the inverted fragment and not seen at microscopic examination because it was immediately underneath the surface exposed to the objective. After transplantation the typical growth was resumed by the two sister cultures.

Extent of Growth

It must be made clear at this point that the increase of total area of the culture does not represent true growth. It does not indicate whether the increase is due to simple migration of cells or to an increase in number, nor does it take into account the increase of the culture that may express itself in greater density or thickness. It has been pointed

out by Fischer and more recently by Parker (1938) that in order to have a complete estimation of the activities of cell colonies in vitro it is necessary to take account of surface-area increase expressed as a function of time, the mitotic coefficient, the weight of the cultures, and the energy production as a result of respiration and glycolysis. But, though the graphs of area-increase do not account for every activity of the respective cell colonies, they are, nevertheless, significant in that they show the general tendency of the behaviour of the culture if not true growth rate.

The difference in behaviour of normal and tumour cultures becomes apparent immediately after transplantation. The factors responsible for the latency of cell proliferation in normal cultures have been pointed out and discussed above. Even when the comparisons with the tumour cultures are made from the time at which the area-increase of normal cultures becomes first apparent, the rate of its area-increase is much slower than that of tumour cultures: the normal cultures usually attain in 144 hours the typical 96-hour area-increase of tumour cultures.

The different behaviour of the two tissues is more striking when the relative daily increment is estimated (Text fig.1-B). The sharp peak in the line representing the relative increments of tumour cultures is due to the fact that in most of the tumour cultures the plasma clot is liquefied about that time and most of the growing "tubules" make contact with the surface of the glass, the cells spreading rapidly in a thin unicellular layer and becoming markedly flattened. Such an extent of liquefaction is reached by normal cultures only after prolonged culti-

vation without transplantation. Normal epithelium does not have the ability to infiltrate plasma, therefore it grows best over the surface of plasma or preferably over a substratum of fibroblasts. This is very similar to the behaviour of epithelium in vivo and is particularly stressed in the process of repair. The proliferation of epithelium usually follows the formation of granulation tissue which is fundamentally composed of connective tissue elements. If the granulation tissue formation is retarded, the epithelial proliferation is slowed up; the epithelium growth is retarded when the granulation tissue matures into scar tissue. The precise interaction of the two tissues is not known; probably the surface tension forces account for some part of this phenomena.

Fischer and Willmer as well as other workers who used tissue culture work extensively observed that cellular activity is subordinate to the activity of the whole colony which in vitro behaves as a primitive organism. Ephrussi (1933) who studied growth and regeneration of fibroblasts cultures, has shown that the characteristic arrangement and organization of a colony is inherent in its heterogenic material, and the limitation of size of a colony and the character of its shape are the result of intercellular interaction, simple diffusion, group movement of cells and products of metabolic activity. The radial arrangement of columnar cells in a growing tumour "bud" has been described. When the buds spread out into a membrane growth the variation of size and shape of cells becomes more marked. Conspicuous intercellular spaces have been observed between the walls of the cells and the intercellular cytoplasmic connections. Normal kidney epithelial cells are surrounded by a homogeneous substance. The tadpole kidney showed a loose cellular structure. Such differences in organization indicate a difference in metabolic activity.

Metabolic Properties of Normal and Malignant Cells.

Malignant cultures were composed entirely of tumour cells. Pure cultures of normal kidney epithelium were not obtained and the comparison of metabolic activities is thus made between tumour cells and mixed components of the frog kidney - primarily epithelial and fibroblast cells.

(a) Respiration

Direct break-down of sugars to CO_2 in air by tissues is one means of obtaining energy. Aerobic glycolysis is the ability of tissue to form lactic acid in the presence of air and anaerobic glycolysis, in absence of air. Cyanide reduces respiration but not glycolysis; the latter is inhibited by the addition of Iodoacetate.

We have observed that addition of $\frac{1}{1000}$ molar KCN to normal and malignant cultures results in an arrest of growth of normal tissues and in partial inhibition of area-increase of tumour cultures. Since cyanide is believed to exert its depressing action on cellular respiration by combining with the oxygen transport system within the cell, possibly with the cytochrome of the cytochrome-oxidase system of Warburg-Keilin (Commoner 1939), the results of our experiment suggest that the malignant cell possesses some other mechanism which can be resorted to when the Warburg-Keilin system is inhibited. The discussion of whether the basis of the effect of KCN is upon the enzyme cytochrome oxidase or, as Potter suggests on the basis of spectrophotometric observation, on cytochrome c is beyond the scope of this paper. The net result is the same: reduce the amount of molecular oxygen. It appears, therefore, that tumour tissue

has a mechanism different from that of normal tissue and more independent of molecular oxygen.

The addition of Iodoacetic acid produced a marked reduction in the area-increase of tumour cultures. Rapkine (1930) proposed a theory that carbohydrate metabolism and sulfhydryl groups are fundamental to cell division. He suggested that reduction of oxidized glutathione (-SS-) by a free -SH group, released by denaturation of certain proteins as a result of protein katabolism, stimulated glycolysis and provided energy required for cell division. Addition of Iodoacetate inhibits the carbohydrate metabolism and also destroys the reduced glutathione. It is impossible to say whether or not the reduction of relative area increase is due directly to the slowing up of the rate of cell division. But the experiment indicates that the reduction of carbohydrate metabolism with $\frac{1}{1000}$ molar Iodoacetic acid is greater for the tumour tissue (in terms of area-increase) than the inhibition of respiration with KCN in the same concentration. The effect of Iodoacetic acid on the normal cultures has not been conclusive and should be repeated.

Snake venom reduced the relative area-increase of tumour more than that of the normal tissues. The reduction in tumour cultures was to about the level of the area-increase of the cultures treated with Iodoacetic acid. Chain who studied the effect of snake venom and respiration reported that in small concentration it inhibits the glycolysis and fermentation of cell-free extracts. As the result of the study of the effect of venom on oxidation- reduction enzymes, Chain concluded (1939) that snake venom inactivates the coenzymes necessary for the activity of dehydrogenases. The cozymase-inactivating factor which is

responsible for inhibition of glycolysis, fermentation and dehydrogenasis requiring coenzymes was shown to belong to the class of nucleotidases.

In the light of Chain's conclusion it appears that the greater reduction of the area-increase in tumour cultures than in normal is due to interference of venom with the glycolysis and fermentation. The results of the experiments design to test the effect of KCN, Iodoacetic acid and snake venom suggest that (a) both normal and tumour tissues are dependent on respiration; (b) normal kidney depends almost entirely on respiration as the source of its energy; (c) tumour tissue has a high glycolysis and is more dependent on glycolitic process than is normal tissue.

In frog tumour tissue carbohydrate metabolism as a source of energy is predominant and in this respect it is similar to embryonic tissues. The similarity is inherent in the rapid growth and proliferation which is characteristic of the two tissues. The effect of respiratory poisons on the normal frog kidney and malignant tissues suggests that the primary difference between them is in the nature of carbohydrate metabolism and the role of Warburg-Keilin system in respiration. Experiments of many workers who studied the oxygen consumption of carcinomata and normal tissues with Warburg's method did not yield any conclusive information which might explain the abnormal growth rate of the malignant tissues. Voegtlin et al (1925) who attempted the quantitative estimation of the reducing power of normal and malignant tissues by means of oxidation-reduction indicators also failed to obtain such information .

The inhibitory effect of spleen extract on tumour was described above. It was noted that $\frac{1}{1000}$ molar KCN restricts the growth of spleen tissue to approximately the same extent as it does the tumour cultures. Though both have high carbohydrate metabolism, the inhibition of spleen extract may be due to the antagonistic action of the glycolyzing enzymes of spleen on the glycolysis of tumour or on its metabolic processes in general. The effect of tumour extract on spleen cultures should be tried to see whether the two have mutually antagonistic glycolytic or other metabolic enzymes.

(b) Protein Synthesis

It is impossible to say specifically, what particular ingredient of the media used in the foregoing experiments or what particular protein or feature of the metabolic change is directly responsible for a given response. Although the experiments were conducted with the utmost care, to obtain uniformity throughout, the complexity of the processes involved and the small number of tests made, due to limitation of time, rendered the task of offering an all-inclusive and definite conclusion impossible. The following paragraphs are speculative, based upon the evidence obtained and are offered merely as a working hypothesis with suggestions for further investigation.

In media lacking frog serum greater relative increase was observed in the tumour tissue than in normal. The mechanism involved in protein synthesis is rather complex. It is believed that proteins are obtained by synthesis from amino acids but there are a host of very important intermediate products. For instance, chick embryo extracts by virtue of the globulin that they contain (and some other substances) make it possible for a tissue in culture to utilize Witte's peptone prepared

from commercial fibrin and used in our synthetic feeding solution. Analyses of the extract (Willmer,Fischer) revealed the presence of polypeptidase, dipeptidase, amylase and lipase. The thermolability of the extract is probably due to the presence of the SH- groups. Thus, in identical media,that tissue will show best growth, which has the most efficient means for protein synthesis.

The difference between normal and malignant cells, if we are to consider all aspects of their behaviour, metabolic reactions and their functional or "functionless" activity, lies in their respective metabolisms. In our experiments it was observed that tumour cultures showed a higher intensity of growth than normal kidney in absence of frog serum (Table I). Baker (1935) has shown that serum must be used with protein degradation products regardless of the degree of hydrolysis. The tumour cultures, however, showed almost the same growth whether the serum was present or not; apparently the protein synthesis mechanism of tumour tissue is more efficient or different from that of the normal.

It would be interesting and important to determine what component of frog serum is responsible for the particular effect on normal and tumour tissues; various available substances found in serum should be tried.

An alteration in protein synthesis is inherent in a different oxidation-reduction system. That tumour is less dependent on the presence of aerobic oxygen has been shown by many workers. The reduction of relative area-increase of tumour culture by the addition of KCN was considerable, but not as great as that of normal cultures; therefore, our experiments support the same conclusion.

It has not been proved that carbohydrate resynthesis occurs in tumour tissue. Burk (1937) analysed the significance of Pasteur reaction and Meyerhof cycle in the intermediate carbohydrate metabolism. He concluded that the evidence concerning carbohydrate synthesis in tumour and other tissues with high carbohydrate metabolism (as suggested by Meyerhof) is not convincing. But Burk suggests that "the problems of energy transfer and resynthesis involved in Meyerhof cycle are doubtless similarly involved in growth and metabolic processes generally." He attributes the normally observed Meyerhof's oxidation quotient in tumour metabolism not merely to resynthesis of higher carbohydrates but to "various other types of synthesis" which are not known.

The tumour has mixed metabolism and its aerobic respiratory quotient is like that of muscle cells, while its glycolytic character is like that of nerves and embryonic tissue. Frog kidney has a very low anaerobic glycolysis and thus ammonia which results in the protein break-down cannot be recombined with the ferment products since the product of its oxidation is CO_2. In tumour, on the other hand, it is possible that the ammonia which is a result of the deamination and dehydration of proteins, can be recombined with fermentation products into amino acids, and in this manner provide more building material.

It is idle to consider the problem of the thermodynamics because the tissue culture methods are too crude, and the study of such processes is limited. With the means now at our disposal the thermodynamic phase of this problem is not likely to be solved.

A change in carbohydrate metabolism of normal cell in the direction of greater fermentative ability may account for the fact that the protein

break-down products can be recombined into amino acids. The nature of such change or the agent that may bring it about is not known. Perhaps it is some autolytic enzyme or substance, demonstrating itself into tumour as a catalyst while still retaining its autolytic nature, which is transmitted from cell to cell. Such enzyme may be directly "injected" with a virus, living or non-living, or by carcinogenic substance, or released within the cell by x-ray or other agent. The action of such "enzyme" may be likened to a nutation which has a dual effect since it demonstrates itself in a different chain of processes as well as reproduces itself.

pH and Other Factors of Metabolism

The results of our experiments designed to test the effect of pH on normal and malignant tissues are somewhat different from the observations of other workers. Maver et al (1935) who described the experiments designed to ascertain the optimum of pH range for the reversal of proteolysis under increased oxygen tension, found no qualitative difference between the nitrogenous metabolism of malignant and normal tissues. The evidences of their experiments in vitro suggest that the lytic activity of tumour proteinase may be increased by excess lactic produced or accumulating during aerobic glycolysis.

However, the work of various investigators suggests that the sulfhydryl group can activate the lytic function of proteinases. The increase of pH (and oxygen tension) increases the oxidized form (S-S) and in this manner inhibits protyolysis which may even be reversed to protein synthesis.

Maver et al found a similar response of the proteolytic enzymes in digests of normal and tumour tissues to changes in pH. But the greater acid condition of the tumour which was noted in their experiment is due to the fact that the digests were subjected to oxygen tension much higher than that of living tissue (20 or 100 volume percent O_2).

The comparative study of Maver et al was conducted with normal tissues with high glycolytic metabolism (rabbit, liver and muscle). Normal kidney tissue, however, has an exceedingly low glycolysis. The greater relative area-increase of tumour as compared to normal kidney cultures in pH 8.2 - 8.4 may be due to the greater oxidation of SH-groups which is increased with the rise of alkalinity and is concomitant with greater protein synthesis. In the light of this interpretation it is possible to assume that the comparatively higher alkalinity of the tumour cells themselves, which may be due to recombination of ammonia with keto-acetic compounds (such as pyruvic acid derived from lactic acid - the product of glycolysis and abundantly present in malignant cells) is conducive to reforming of new amino acids. Alanine, which may be resynthesized in this manner, has a considerable initiating and dynamic action (Hammett, 1937; Reinmann, 1941).

The tumour cytoplasm appears to be more alkaline than the cytoplasm of normal cells: malignant cells are less conspicuously stained with vital acid dye, such as Neutral Red. Water soluble acid dyes do not stain fixed tumour cells, or do so very faintly. Whether alkalinity leads to deposition of fat and lipoids on plasma membrane and thus renders the cells impermeable to acid dyes has not been proved. Yasuda and Bloom (1932) have demonstrated that tumour cells do have a much larger percent

of fatty substances than benign, and have particularly high phospholipoid content. We have observed that vacuolation of cytoplasm in tissue cultures is very marked in acid media. Evidently the increase of acidity either renders the cell membrane more permeable to certain substances or changes the metabolic activity. It is thought that the increase in acidity in areas adjacent to regions of injury increases the permeability of blood vessels - admittedly a different kind of permeability - and facilitates the "pavementation" and emigration of leucocytes through the vascular endothelium.

Hypotonic solutions and substances that exert surface action are stimulating to growth. The effect may be due to increased permeability which facilitates the exchange of fluids with the surrounding medium. The mechanism of this action is not well understood. Most of the authors dismiss this problem by saying that the colloids of embryonic and rapidly growing malignant tumours are in a state conducive to growth and to continuous cell division.

Kopaczewski (1935) points out that a number of compounds with carcinogenic activity lower the surface tension of serum. It is possible that similar forces are in action around a malignant cell: the constant activity of the ruffled margin of the cytoplasm around tumour cells bespeaks an interplay of surface forces. We have not measured the quantitative difference of permeability between normal and malignant cells nor the different forces at work at the periphery of the cell membrane. However, we have observed that malignant cells have a greater variation of surface tension; such activity has not been seen in normal cells.

Although the surface tension of the covering cell membrane and the ionic charges have an effect on the interfacial phenomena, they are the result of the changes taking place in the colloidal state of the cytoplasm. The cytoplasm is a complex dynamic system which does not stay constant for any appreciable length of time. Constant alteration of sol-gel phases are evident in the living cell at all times though the rate of the interaction may vary with different tissues. In all cells during mitosis these alterations are more pronounced. In rapidly growing tissues the sol-gel alteration occurs at a greater rate. Normal kidney cell which is a comparatively slow growing one does not show as much marginal activity as do the malignant cells. The measurements of the surface tension of the tumour and normal kidney extract should be made. This direct method may reveal the characteristic differences of the two tissues.

The tests of the permeability can be carried out in vitro by placing the tissues in various media and observing the volume change in a graduated capillary outlet. Such experiment should be done with normal and malignant tissues. Other tests bearing upon the specific effect of different isoelectric points, surface tension effecting substances, and other pertinent agents should be attempted.

Cytology of Normal and Malignant Cells.

The most detailed descriptive analysis of the cell may be interesting but it is significant only when it can be interpreted and correlated with the functional, metabolic and physiological nature of behaviour of the cell. The shape of cells cannot be used as a criterion for identification of cells unless the conditions of growth and the media are identical, because the cell shape is almost directly influenced by the state of the surrounding medium. Our comparisons were made between normal and tumour cells cultivated in some media and correlated with the study of sectioned material.

Lewis (1941) pointed out that the extent of visible change that distinguishes tumour cell from the normal cell is not a criterion of the degree of their malignancy. The change is significant in that it may outwardly demonstrate that an alteration has taken place, and may sometimes give a clue to its nature. It was pointed out above that frog tumour cells are generally larger than normal kidney cells; the tumour nucleus is generally denser and contains one or two conspicuous nucleoli. The mitochondria are more filamentous and sometimes more abundant. The cytoplasmic connection persist longer between moving tumour cells and extend over large distances. Unlike normal kidney cells which have a wavy cell outline, tumour cells have an undulating margin of ruffled pseudopodia. Tumour nucleoplasm appears to be more granular than normal in living cultures, though when stained the normal nucleus shows more nucleo-chromatin. These differences from normal are general for almost all malignant cells (Lewis and Lewis, 1941).

Tumour cells in locomotion ingest globules of media by the action of their ruffled pseudopodia. This phenomenon is known as

pinocytosis and is characteristic of tumour cells and of macrophages but not of other normal cells. Lewis (1937) pointed out that the advantage of pinocytosis is that substances with molecules that are too large for diffusion into the cells may be taken into the cytoplasm in this manner. The explanation is probably oversimplified. The observations seem to indicate that portions of the medium are incorporated into the cell, but whether the globule is in direct contact with the cytoplasm or is still surrounded by a portion of cell membrane which was pinched off, is difficult to say. The advantage of pinocytosis is obviously in the increase of surface that is in contact with the medium, and this fact may facilitate assimilation of nutrient material. Moreover, pinocytosis is observed only in isolated tumour cells and its significance in the tumour growth in vivo is not great.

The universal characteristic of all malignant cells is their uncontrolled growth. But since they are seldom uniform in properties we will confine our discussion to such features that are characteristic of all malignant cells. Lewis and Lewis (1941) offer the following characteristic and properties of malignant cells. Malignant cells are permanently altered ones, producing an uncontrolled growth in the body. They are transplantable to other animals of same strain. Some have ability to invade other tissues. They have peculiar cytological characteristics which distinguish them from the normal cells; these characteristics are maintained in vivo as well as in vitro.

The frog tumour possesses all of these characteristics. It is an adenocarcinoma arising from the kidney tissue which it resembles in the manner of growth. The ability to metastesize is present in large tumours. The nature of its growth is affected

by the environment in which it grows: in vitro the growth may be cylindrical or membrane like depending upon the substratum. The same types of tumour growth were observed in our experiments as those obtained by Lucke and Schlumberger (1939a) in intraocular transplantation.

The difference between normal and malignant tissues are focused upon the chain of factors that make up the essence of metabolic activity: the mechanism for protein synthesis, may be effected by the oxidation - reduction mechanism; both in turn may account for the state of cytoplasm within the cell and its iso-electric point. All three effect a change upon the membrane of the cell. The permeability of the cell membrane is controlled by the deposition of materials and its ionic charges at the surface, and is also affected by the acidity or alkalinity of the environment. The foregoing gradation is somewhat schematic because metabolic processes have no sharp separation and are concurrent, but we suggest it in a simplified form, basing the conclusion on the results of the experiments discussed in this paper. All cyto-logical differences from normal cells are better understood in the light of these metabolic differences. The anabolic processes of malignant cells exceed the katabolic. The malignant cell reaches the optimum size in less time than normal and must divide. But, due to more rapid metabolic processes, alteration in colloidal state of cytoplasm are more drastic (ruffled pseudopodia, pinocytosis of resting cells). The interplay of streaming and gelation are likely to produce chromosomal abberation or incomplete mitosis which gives rise to binucleated cells, or upset the centerosphere into a tripolar division.

VI. Summary.

1. Normal adult kidney and adenocarcinoma tissues of the frog, Rana pipiens, were cultivated in homologous and heterologous media. The different manner of growth was observed in these tissues. Tumor explants begin to show growth in the form of cylindrical protrusions which spread out into a unicellular membrane when contact with the glass is established. In the normal kidney cultures the infiltration of the fibroblasts serves as a substratum for the epithelial proliferation.

2. The malignant cultures showed a greater relative area-increase than the normal. The difference is due to a greater rate of growth of tumour tissue and to the period of latency which follows each transplantation of normal kidney cultures.

3. The metabolic properties of normal and malignant tissue were studied by subjecting the cultures to the action of respiratory poisons, various tissue extracts, changes of temperature and of pH. The effect of these changes on the behaviour of normal and tumour cultures was determined by measurements of their relative area-increase.

4. KCN, high alkalinity, and absence of homologous serum slowed up the rate of area-increase of the normal to a greater extent than that of the tumour cultures.

5. Snake venom, spleen extract and low pH produced a greater retarding effect on the tumour than on the normal cultures.

6. These experiments indicate that tumour and normal tissues have different oxidation-reduction and carbohydrate mechanisms. It is suggested that alteration in carbohydrate metabolism is concomitant with the changes in the mechanism for protein synthesis.

7. Experiments designed to test the specific metabolic properties of normal and malignant tissue were outlined.

8. The cytology of normal and tumour cells has been described. The cytological differences were discussed in their relation to the probable alteration in metabolic properties of the two types of tissues studied.

VII. Acknowledgements

The writer wishes to record his indebtedness to Prof. N.J. Berrill and to Prof. C.L. Huskins, under whose direction this problem was studied, for encouragement and advice. Acknowledgement is also made to Dr. R.W. Briggs for many useful suggestions on the scope and possibilities of the problem.

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IX. Description of Plates

The figures are unretouched photographs of living or fixed cultures. The magnification is approximate.

Plate I.

Fig. 10. Lymphocytes, macrophages fibroblasts and endothelial cells in the neighbourhood of frog kidney fragment. X250.

Fig. 11. Fixed and stained 120-hour culture. A part of the growth has been washed away. Hematoxylin-eosin. X24.

Fig. 12. A sheet of epithelial growth over the surface of plasma. The fibroblast growth is at a different focus level.

Fig. 13. Normal kidney culture: a part of epithelial growth proliferating over the substratum of fibroblasts. Hematoxylin-eosin. X160.

Plate II.

Fig. 14. Margin of tumour fragment 72 hours after explantation advance shape of budding formation showing numerous convolutions. X120.

Fig. 15. Living culture of tumour - 96 hours of incubation. Fusion of streaming fan-shaped growth of cells.

Fig. 16. An undifferentiated unicellular layer of tumour cells. The cytoplasmic intercellular connection between the cells is seen. X240.

Fig. 17. Living tumour - 120 hours. Fourth transplantation. The membrane gland adheres to the glass and is not in sharp focus. X50.

Fig. 18. 72 hour tumour culture; spherical "buds" just before contact with glass takes place. X160.

Plate III.

Fig. 18 and 19. Normal kidney cultures in cobra venom. Note marked vacuolation and more transparent cytoplasm. X320.

Fig. 20. The culture shows irregular sheet formation and vacuolation of tumor cells. The outline of cell margin is very sharp. The outline of the whole fragment is seen in one corner.

Fig. 21. Tumor cultures in M/1000 KCN. The bud formation is less extensive in the normal. X50.

Plate IV.

Fig. 23. Living tadpole culture; marked tissue formation is absent. The cells are of mixed types characteristically present in pronephrose. X50.

Fig. 24 and 27. Tadpole cultures, showing marginal cell migration. The cells are of mixed character: melanophores, mesenchymal cells; cells of mesonephric rudiment and melanophores. X240.

Fig. 25. Frog kidney culture subjected to 3°C for 30 days. The culture resumed the normal growth rate when brought to room temperature. X50.

Fig. 26. The fibroblast growth of spleen fragment (larger piece) is seen overrunning a kidney explant placed adjacent to it. 12 day growth. X30.

Fig. 27. Two daughter tumour cells at completion of mitosis. X750.

Plate V.

Fig. 28. Living tumour cell in locomotion. The ruffled margin of cytoplasm shows constant activity. X500.

Fig. 29. An isolated living tumour cell in 96-hour cultures. The nucleus has two nucleoli. Note globules, vacuoles and granules are framing the nucleus and the centrosphere. The margin of cytoplasm is vague.

Plate V.

Fig. 29. (continued) Thread-like mitochondria are seen radiating from the central area. X50.

Fig. 30. A single mesothelial tadpole cell shows dense cytoplasm and thread-like processes. The cell is flattened out against the glass.

Fig. 31. Margin of a fixed and stained tumour culture showing a trinucleate cell. Dense granular cytoplasm, nucleoli and fat globules are conspicuous. Neutral formalin hematoxylin-eosin. X250.

Fig. 32. Tetranucleate cell from a margin of a tumour culture. (Type I medium 144 hours) Discrete globular structures, granules and mitochondria can be distinguished. The margin of cytoplasm is vague.

Fig. 33. A marginal area of tumour culture. (Type I, medium, 144 hours) The field includes the cell shown in Fig. 32. Hematoxylin-eosin. X250.

Fig. 34. An area of 144-hour tadpole kidney culture showing a flat mesothelial, 2 macrophages and one melanophore. X500.

Fig. 35. Single marginal tumour cell showing two nucleoli. The margin of cytoplasm can be made out with careful examination. Hematoxylin-eosin showing culture 144 hours. X900.

Fig. 22. (Plate III) Membranous tumour growth in 1/1000 molar KCN. The cytoplasm appears more granular. The nuclei are pycnotic in some cells.

Fig. 27a. (Plate IV) Two sister tumour cells at the completion of mitosis. X750.

Plate VI.

Fig. 36. Mixed growth of normal kidney showing fibroblasts, macrophages, lymphocytes and epithelial cells. X160.

Fig. 37. A streaming of undifferentiated membrane from the margin of a normal kidney tubule which shows a typical epithelial arrangement. Living, 168-hour culture. X320.

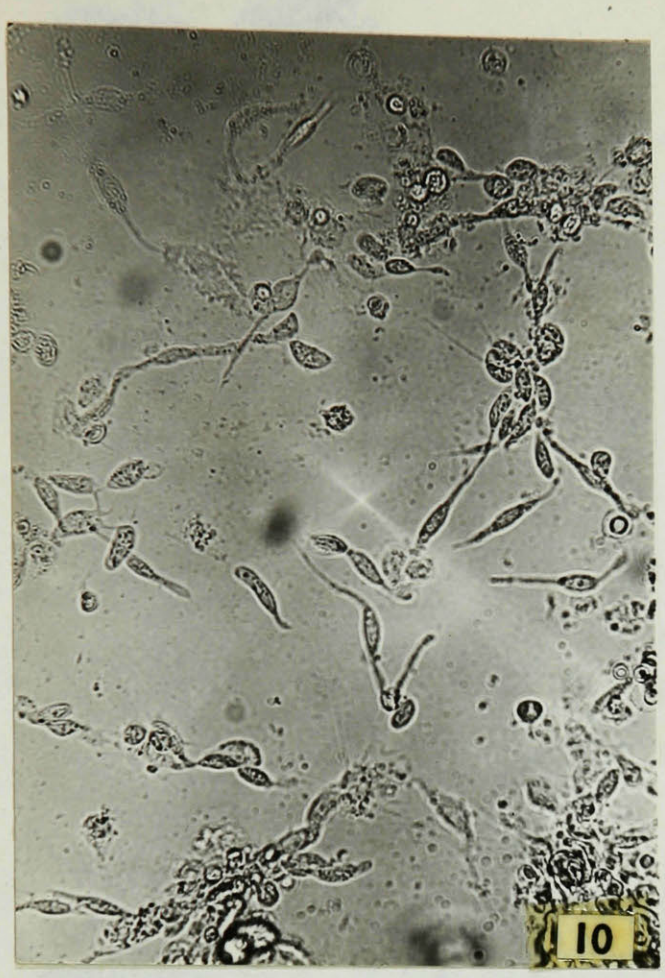
Fig. 38. Shows the flowing end of a tumour "tubule" spreading over the surface of the glass. A single spherical structure seen in the field is a bud which has separated from the fragment. Living tumour. X120.

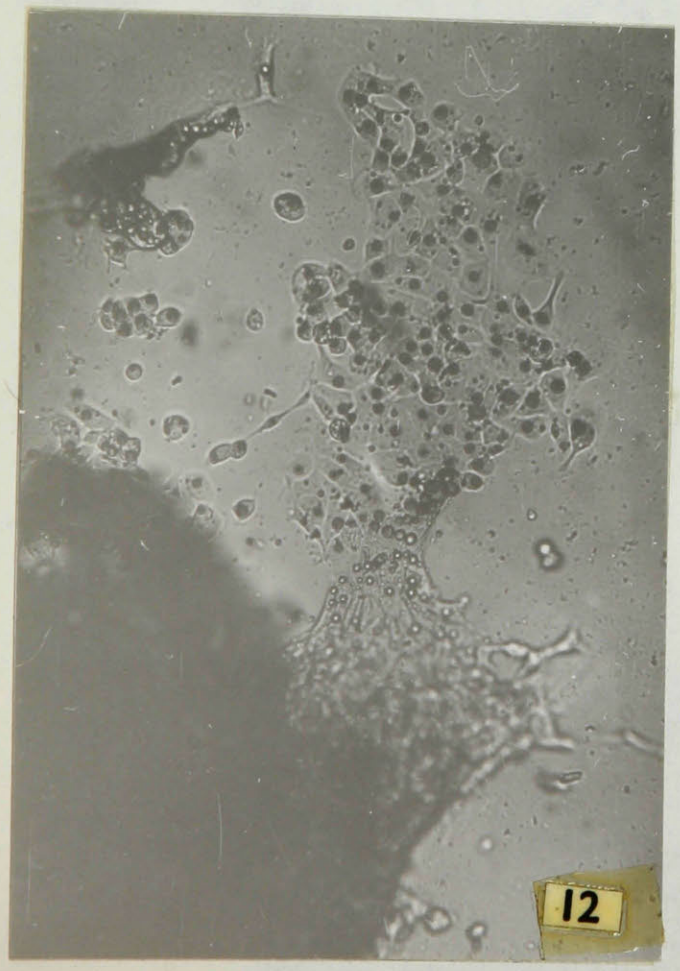
Fig. 39. A cylindrical bud-like outgrowth in 48-hour old tumour culture. Note the high columnar epithelial formation of cells within this structure. No lumen present. X240.

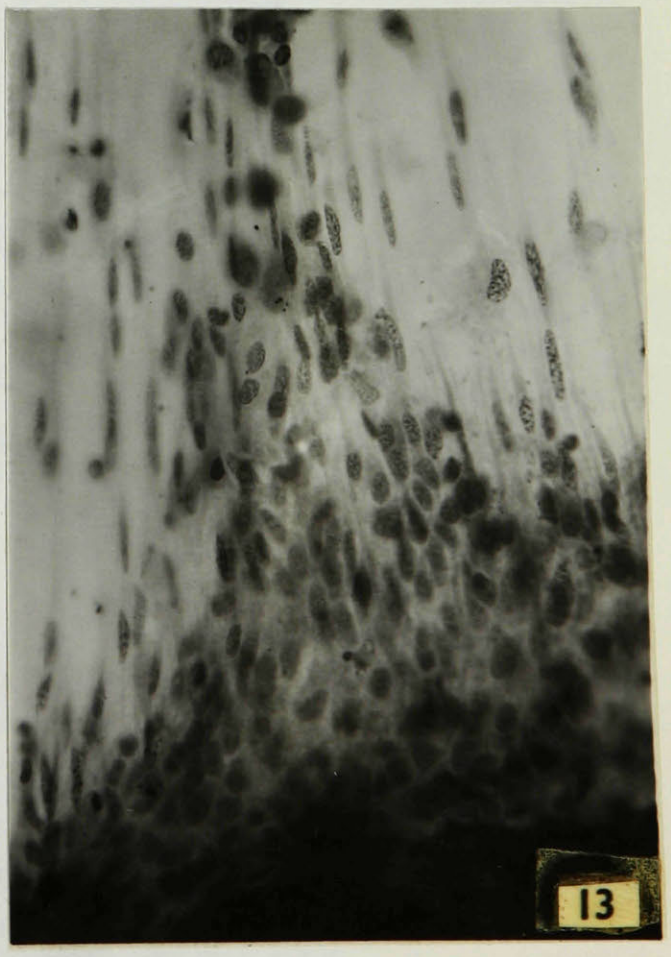
Fig. 40. Marginal area of a 144Ohour tumour culture. The tetranucleate cell shows signs of degeneration. Hematoxylin-eosin. X600.

Fig. 41. Margin of tadpoles kidney explants showing their composite cell structure, dense cytoplasm, and "loose" arrangement of cells in tissues. X500.

PLATE I.







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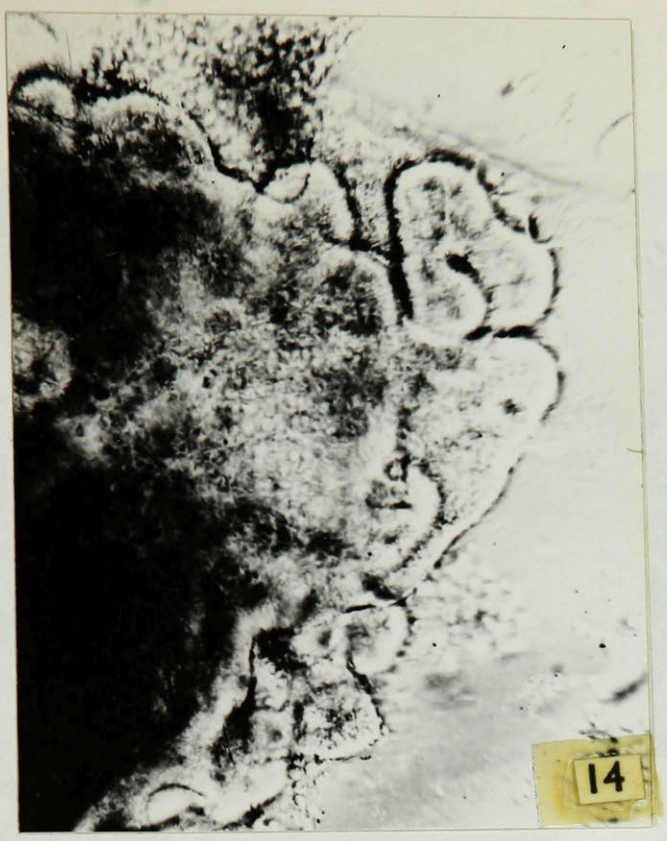

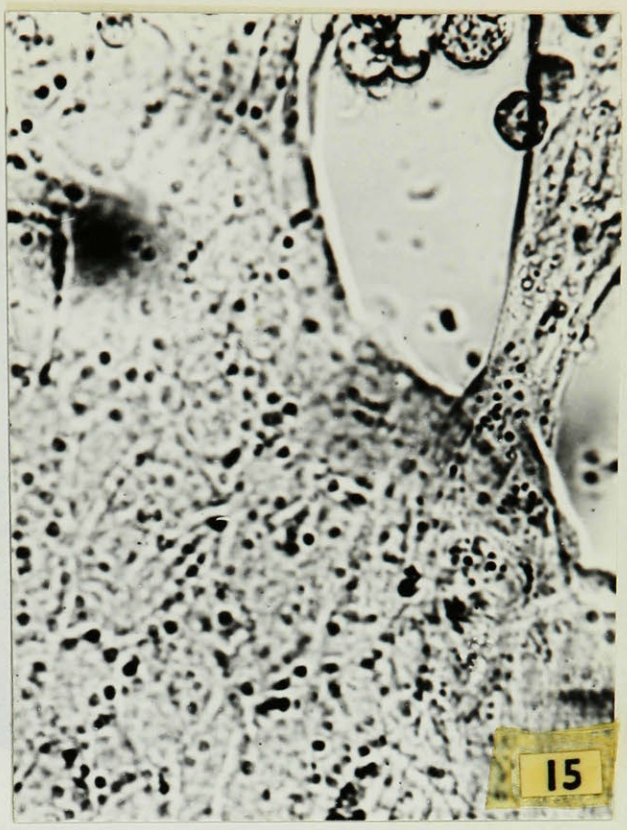

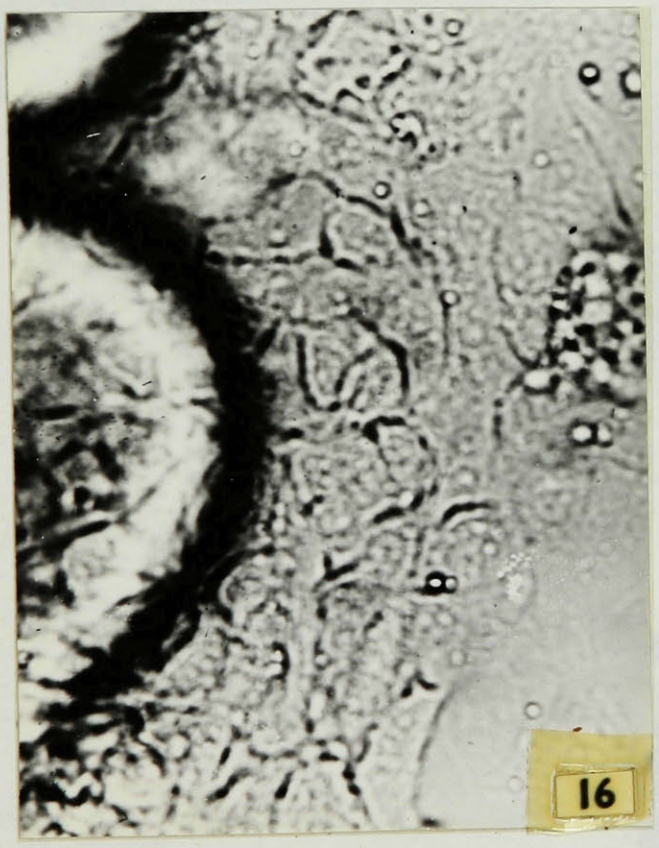

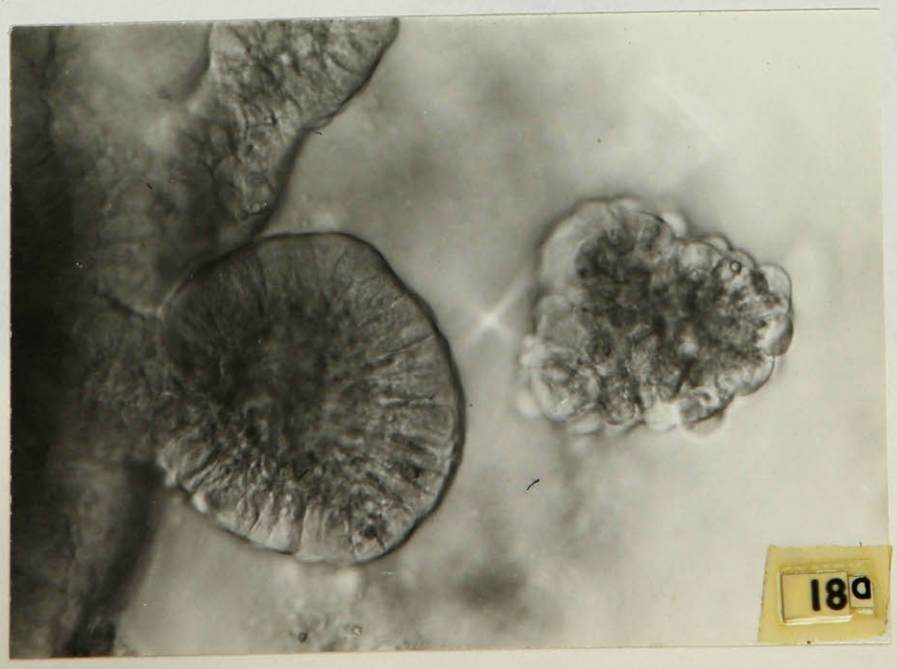

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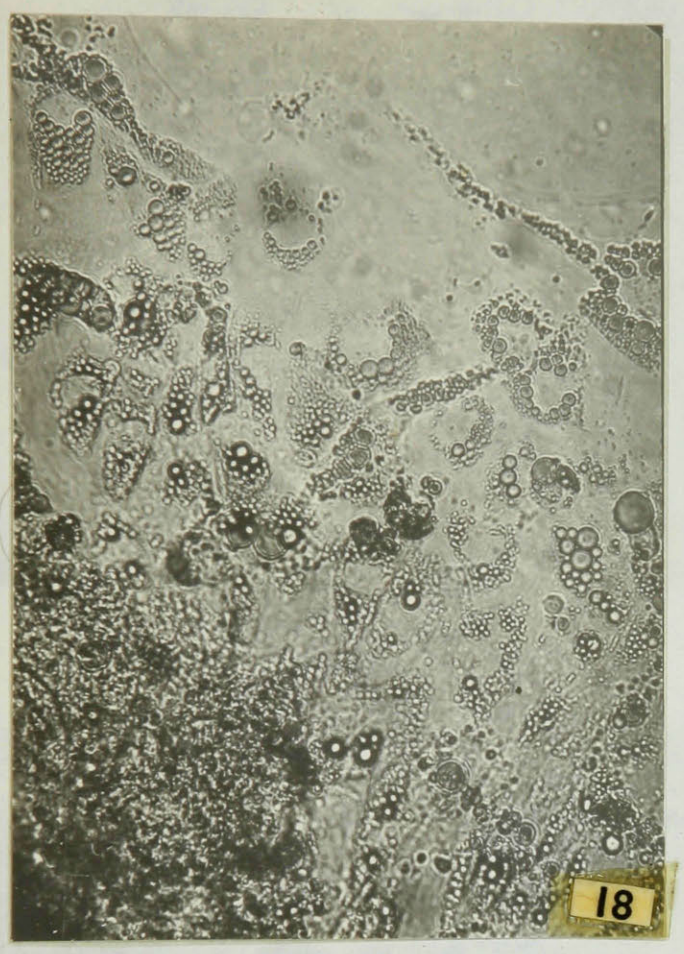

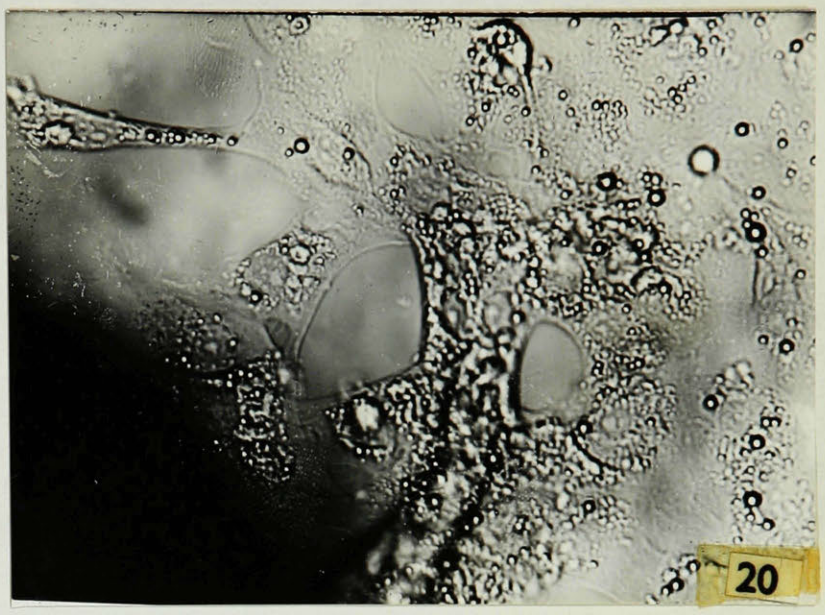

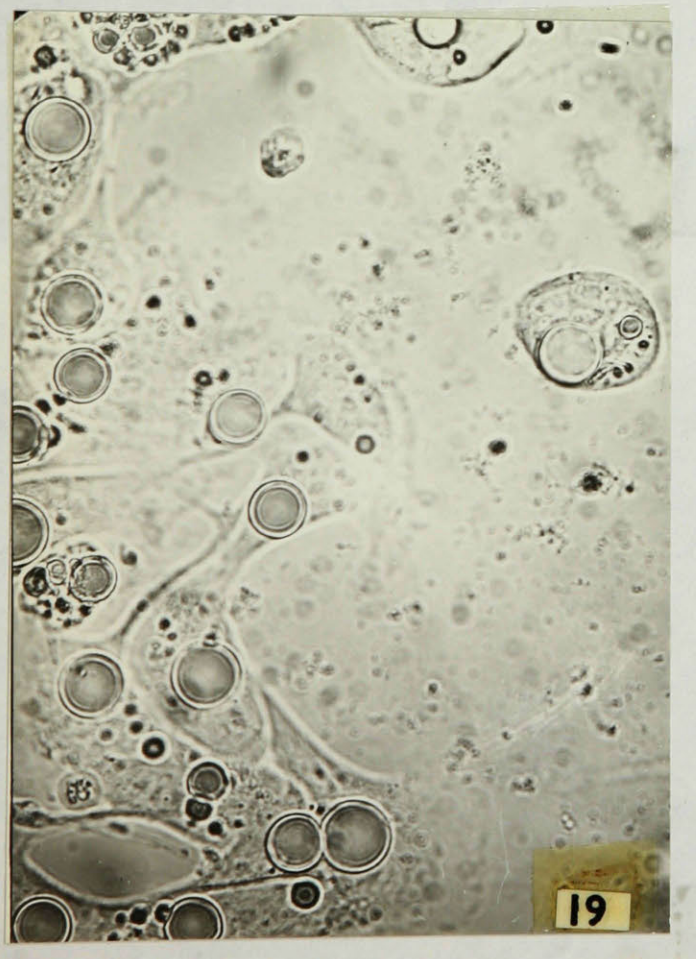

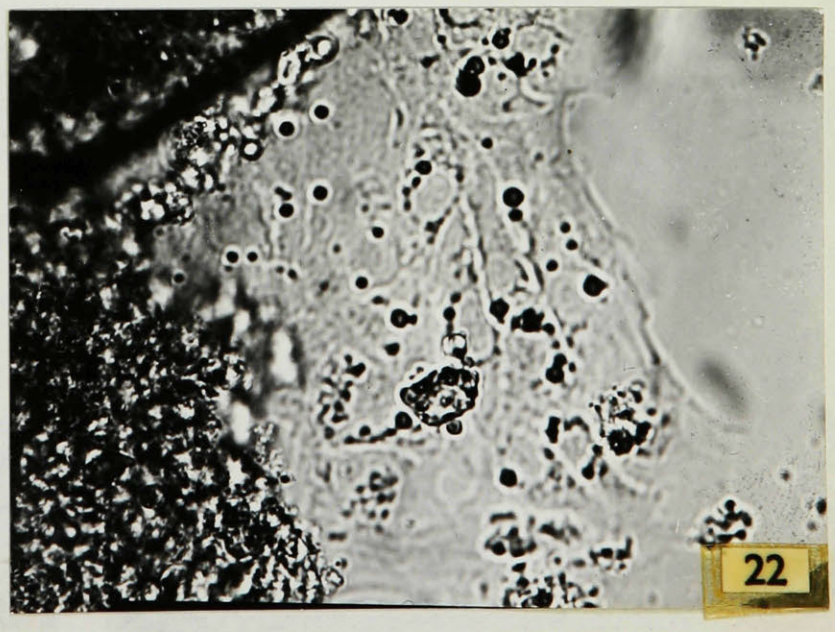

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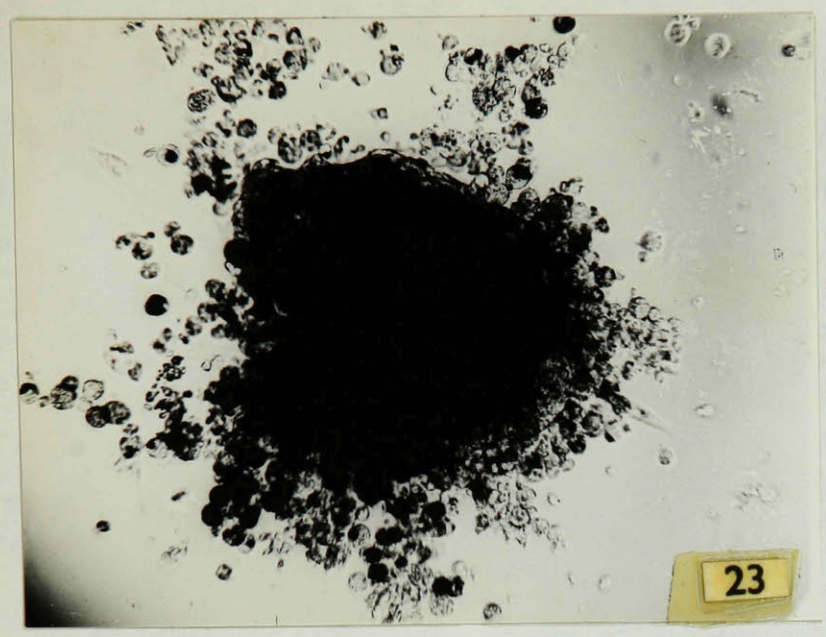

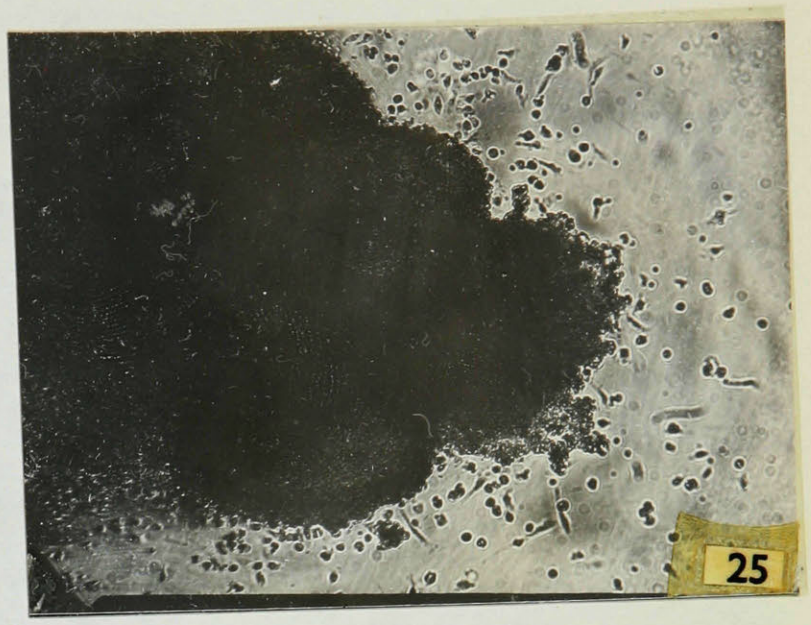

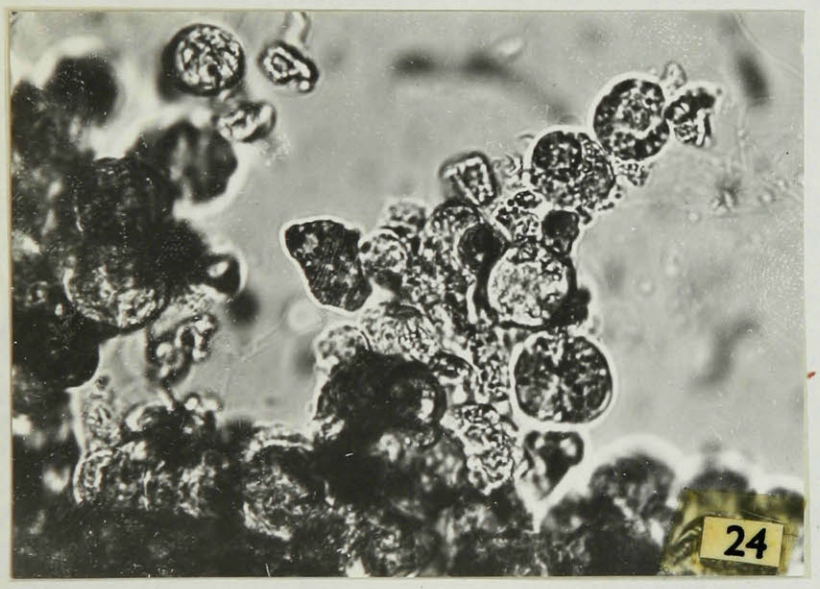

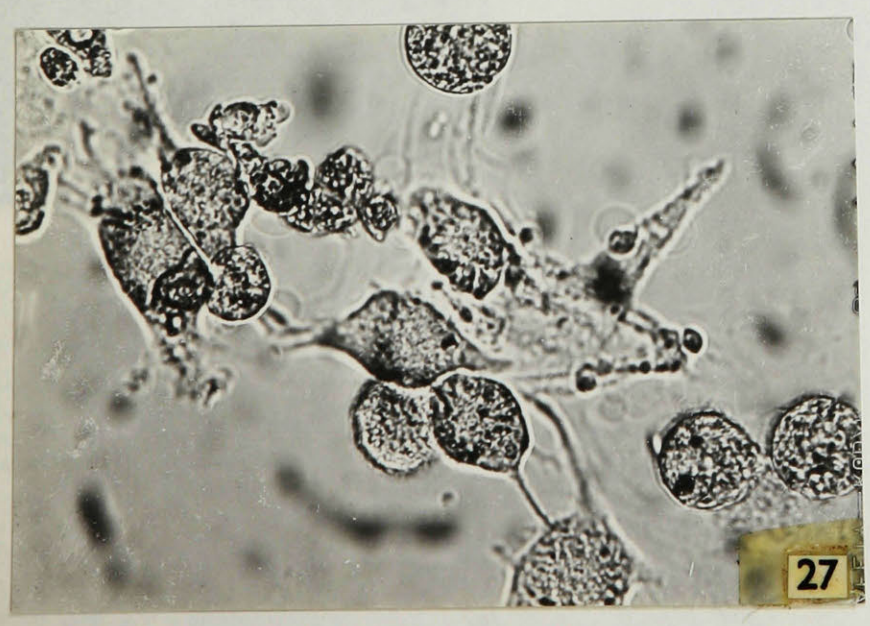

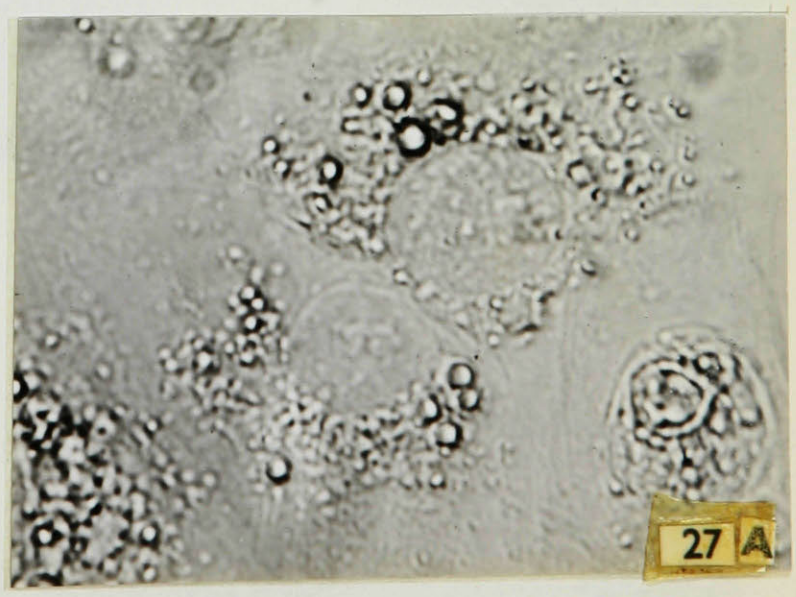

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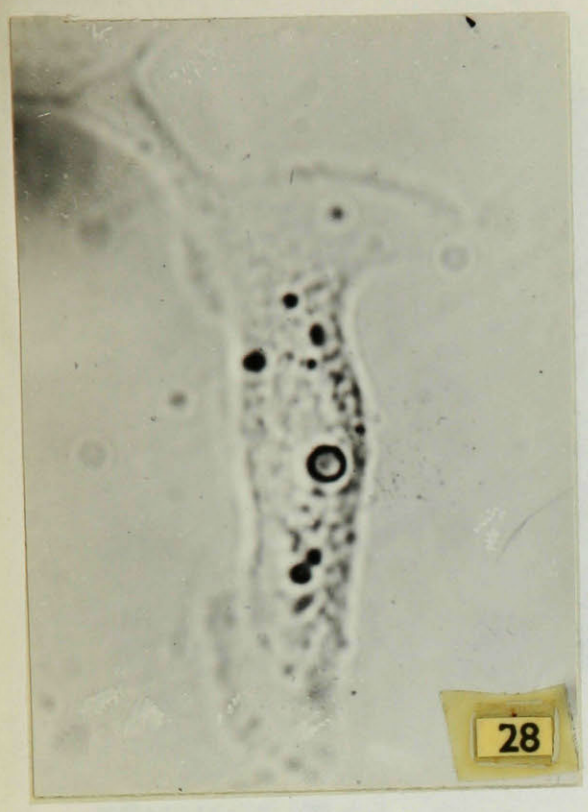

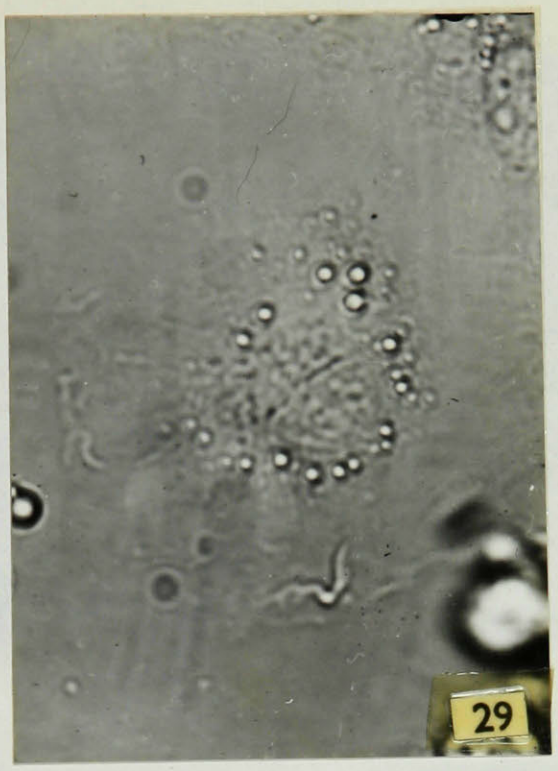

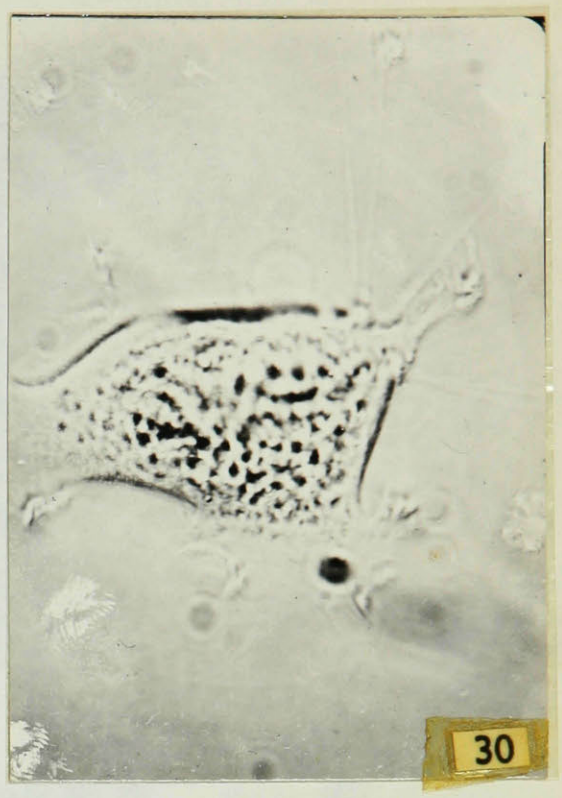

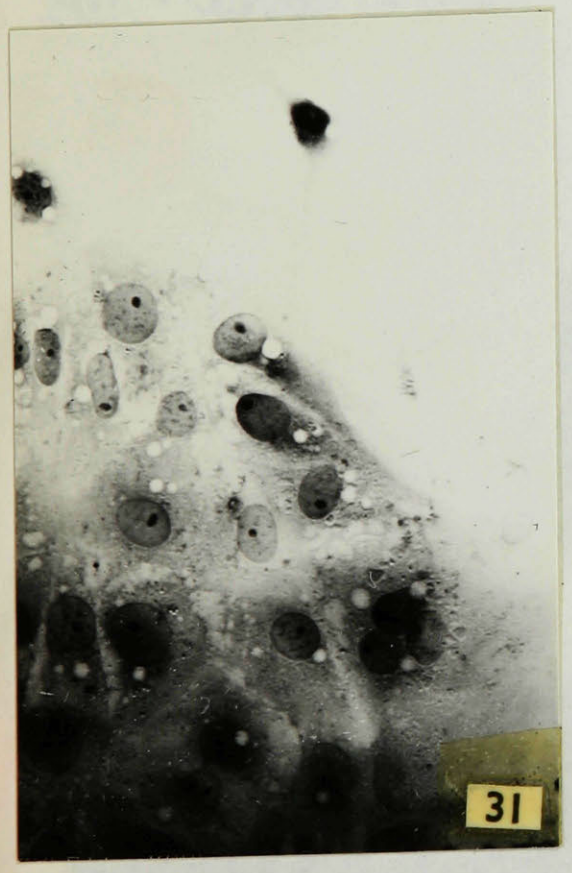

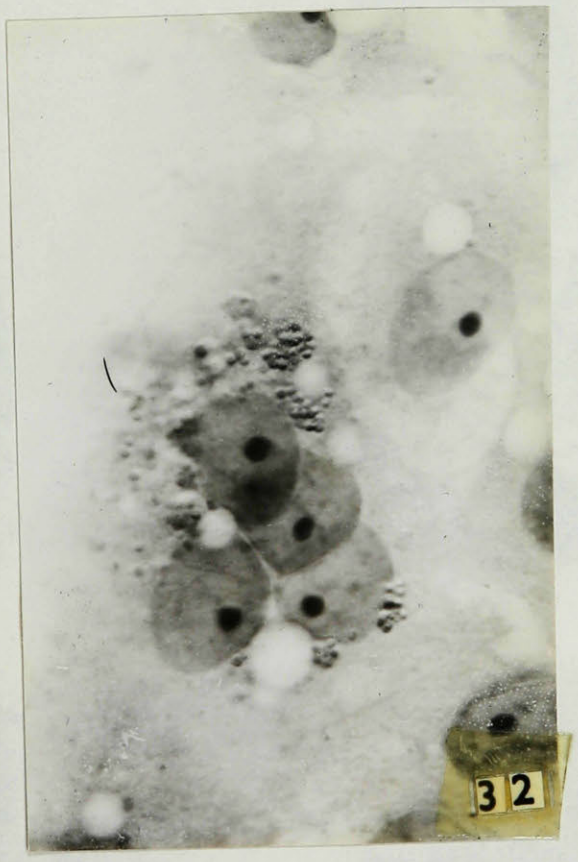

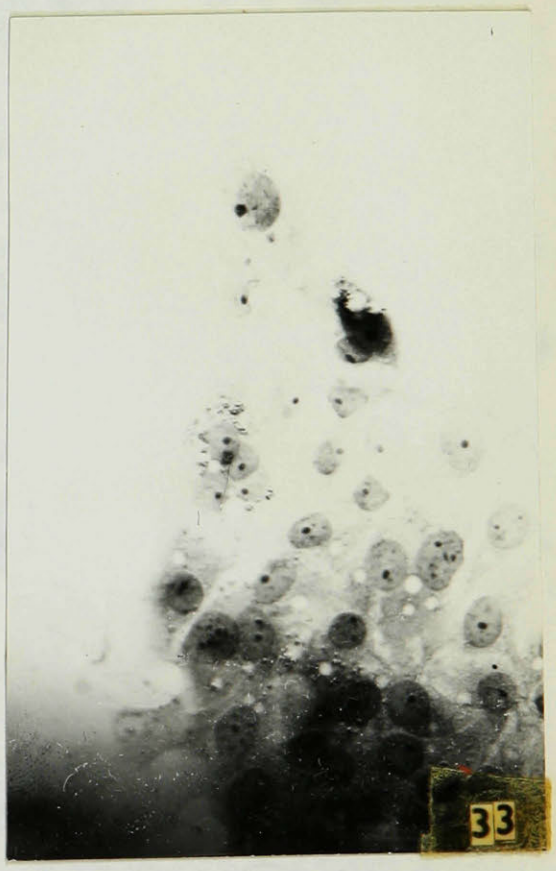

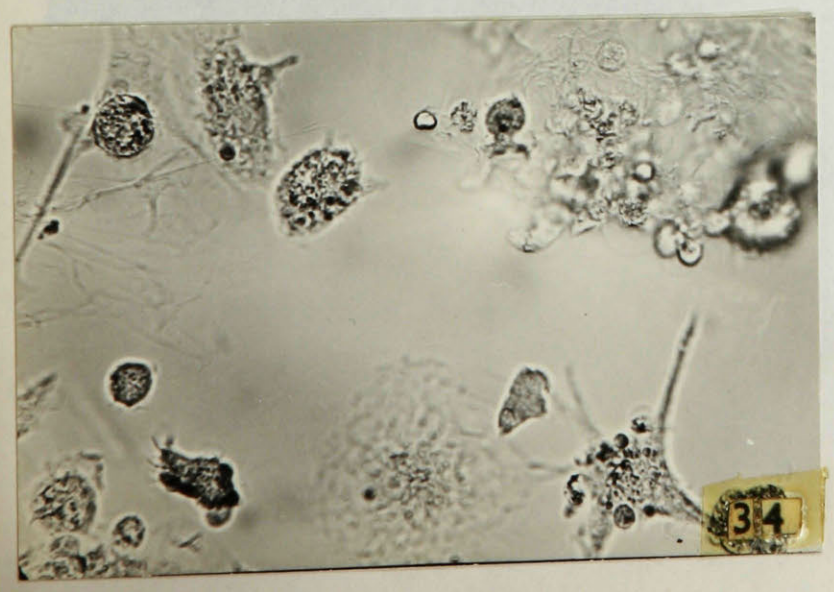

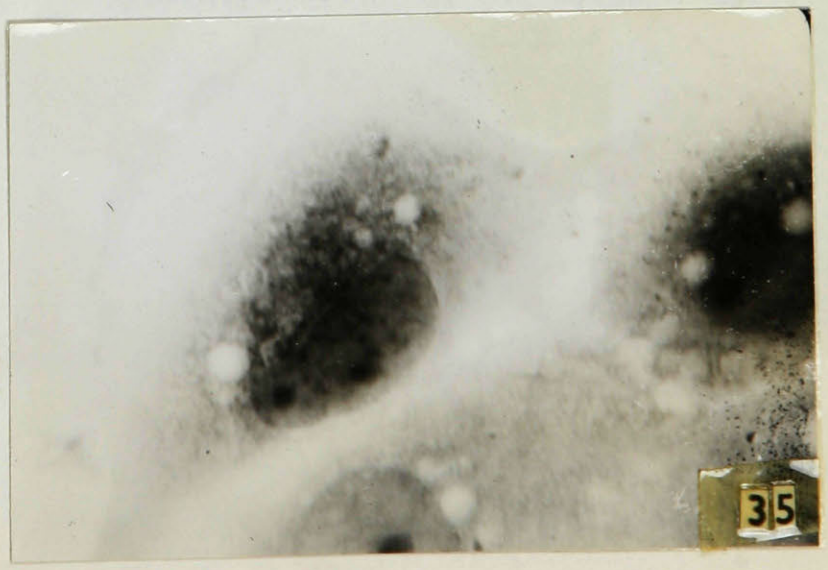

PLATE VI.