MITOCHONDRIAL STUDIES OF HUMAN SKIN CELL CULTURES INFECTED WITH VERRUCA VIRUS

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ABSTRACT

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This study was designed to investigate the effects of verruca virus on the mitochondria of human skin cells and particularly the total number of mitochondria in the cells.

The cells were produced in two types of media but the serum nutrient was the same. The source of verruca virus was fresh wart tissues and tissue stored in 50 per cent phosphate buffered glycerine. Morphological and cytological studies of mitochondria from infected test cells and uninfected cells were done. Cells were homogenized and then fractionated using differential centrifugation methods. The mitochondria were identified by their ability to stain with low concentrations of Janus green B. Comparison of the morphology of mitochondria isolated from the cells and those seen in the intact cells appeared similar. Mitochondria from infected cells were mainly filamentous, elongated or slightly swollen. Mitochondria from uninfected cells were mainly rodlike. Infected cells, regardless of medium, contained more mitochondria than the uninfected cells.

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By

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DEDICATION

This work is dedicated to my parents.

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INTRODUCTION

The human wart virus from verruca vulgaris, a member of the papova group, is related to polyoma and papilloma viruses. The virus is of considerable interest as it is the only virus known to produce tumors in man. The tumors sometimes become malignant. The verruca virus is structurally similar to the polyoma virus which produces malignant diseases in animals.

The viral causation of malignant tumors in man is at present a theory and rests only on indirect evidence. The best basis for this belief lies in the undisputed role of viruses in malignancies of animals. It is therefore difficult to conceive that the human species would be so unique as to escape infection by viruses which produce tumors.

Infection of a cell with a virus is manifested in many ways. The effect may be observed in the cell, the organ and the complete animal. The most frequent microscopic result of viral infection is necrosis and degeneration, however, this may not always be observed. Also, necrosis and degeneration are the final results of infection. Infection of a cell by a virus produces changes, some of which are not easily determined and may require special methods for their detection. When viruses penetrate suitable cells they multiply at the expense of the cells. Since viruses lack the full physiological and biochemical endowment of cells, they are unable to generate the energy required for their multiplication. Consequently they depend on the enzyme

systems of the infected cells to supply the energy.

The living cell is a very complex unit. It is the fundamental unit of which all living organisms are made. There are many types of cells and the cells of the brain or skin tissue are different in morphology as well as in function. Despite the differences all cells have several things in common. They have a cell membrane, a cytoplasm containing various organelles and a central nucleus. In addition to having some definite structures, cells have a number of functional capacities in common.

The protoplasm of living cells has been studied extensively but much remains to be understood. What was originally considered a structure composed of an external membrane, a cytoplasm and a central nucleus has been shown to be differentiated into organelles adapted to carry on the many diverse processes of life. With the aid of the electron microscope, cell biologists have begun to discern the molecular activities of the parts of the system. In recent years, their work has converged with that of the biochemists who have traced some of the pathways by which the cell carries out the biochemical reactions which underlie the processes of life.

One of the most important components of the cell are the mitochondria. They are the sites of oxidative reactions that provide the cell with the energy it needs for its metabolic and synthetic reactions. The mitochondria are present in all living cells, both animal and vegetable. They are the "power-plants" of all life. Each mitochondrion is adapted to its

function by a fine structure. They extract energy from the chemical bonds in the nutrients of the cell by oxidation and respiration. The energy is generated in the form of the compound adenosine triphosphate (ATP). The ATP in turn stores the energy produced in the mitochondria and supplies it to the cell when needed.

It is also known that the mitochondria are an extremely sensitive indicator of cell injury. It was therefore decided to study the effects of verruca virus on the host-cell mitochondria, particularly as the virus affects the total number of mitochondria in skin tissue cells under various experimental conditions. Other studies included the cytological and morphological characterization of the mitochondria both in the intact cells and in the isolated state.

REVIEW OF LITERATURE

Human Warts and the Wart-Virus

Common warts may be separated into four varieties (1). 1. Verruca vulgaris--the raised warts frequently observed on the hands. 2. Verruca plana juvenilis which are usually found on the hands and face of children. 3. Verruca acuminata which are pointed filiform and cause genital lesions which may become malignant. 4. Laryngeal papillomata. Warts may occur also on lips, in the nostrils, in the auditory meatus or on the edges of eyelids.

Foot (2) describes the common wart as a benign tumor which is usually composed of a number of papillae comprising horny scales and clustered in patches. It may be flat and the papillae inconspicuous or it may resemble a tuft of hair-like projections clustered on a stalk. It may have a cauliflower-like complexity. The papillae may, in some instances, be fused into a hornlike process, attaining a centimeter or more in length and diameter and project from a cupped depression in the skin.

Warts differ from malignant cancers in that they grow outward from the surface of origin, while cancers invariably grow inward (3, 4). Warts are extremely hard due to excessive keratinization of the epidermal cells, have a smooth margin and are sometimes surrounded by an erythematous halo. It is in these type of warts that Bunting et al., (5) reported that the wart virus is commonly found.

It is not known when the infective nature of warts was first discovered. For many years people have known that warts can spread by contact and it is further known that warts can disappear upon suggestion or simply by being wished away even though no scientific evidence exists to suggest that this is possible.

The first experiments reporting the infectious nature of warts were those of Jadassohn (6). His experiments, begun in 1894 consisted of a series of intradermal injections of ground up wart material or wart tissue fragments into both himself and his assistants. Warts resulted in 31 out of 74 sites of inoculation, thus demonstrating that warts were transmissible from man to man. He made no attempt to explain the etiological factor.

The viral nature of the agent involved was demonstrated when Ciuffo (7) produced warts on both his hands and those of the patients from whom the warts had originally been removed. The Berkefeld N filtrates of extracted wart tissue were used and after 5 months incubation period, warts appeared on both subjects. Ciuffo's findings not only confirmed those of Jadassohn but also demonstrated that a cell-free filtrate of wart material was infectious. Serra, in 1908 confirmed the filterability of the wart agent. These findings were further confirmed by Wile and Kingery (8) who were able to induce warts by the introduction of cell-free filtrates. Kingery (9) removed an experimentally produced wart and after filtering the extract, reinoculated another volunteer,

producing again a wart growth. Goldschmidt and Kligman (10) repeated these procedures and were unsuccessful in producing warts with their extracts.

In all the transmission experiments in human beings, a long incubation period, 5 months or more, was required before the appearance of a wart growth. In most instances the warts regressed shortly after their appearance.

In 1953, Bivins (11) removed a wart from his finger, inoculated a broth suspension of the homogenized verrucous material onto the chorioallantoic membranes of 10 day old chick embryos. Whitish "pearl" growths appeared on the chorioallantoic membranes after several days incubation. This agent was successfully transferred in eggs with material filtered through a Berkefeld V candle. He concluded the wart virus caused the massive proliferation and "pearl" formation. Siegel (12) found that the agent isolated by Bivins was a strain of a canary poxvirus.

Siegel and Novy (13) were unable to successfully cultivate the viral agent causing verruca vulgaris and condyloma acuminatum in both the chorioallantoic membrane of the chick embryo and in tissue cultures of HeLa and monkey kidney cells. Siegel and Novy's findings confirmed those of Felsher (14). Felsher (14) could not isolate the virus by the inoculation of the chick allantoic membrane with material from cutaneous lesions.

The first report of the successful isolation of the wart agent was in 1961 by Mendelson and Kligman (15). The

human wart virus was successfully isolated in monkey kidney tissue culture cells. Inoculation of human volunteers with cell-free nutrient fluids from the cultures produced wart growth in the skin of the volunteers. In the same year, Hayashi (16) in our laboratory reported the isolation and serial propagation of the wart agent in cultures of normal human skin. This agent was neutralized by undiluted homologous patient's serum and by human gamma globulin.

Strauss et al. (17, 18) examined concentrated extracts of wart tissues under the electron microscope. "Crystalline" virus-like particles from skin papillomas were characterized by intranuclear inclusion bodies. The common wart and normal skin preparations did not contain uniform particles but merely amorphous scattered clumps of matter, collagen fibers and spherical sizes of varying diameter. The particles in the crystalline array averaged 52 mu in diameter. The findings of Strauss et al. were not confirmed by Siegel (12). The latter, using a refined concentration method, found a large variety of particle sizes. In some of the preparations, uniform 16 mu virus-like particles were observed and Siegel hypothesized that these might be the wart agent.

The electron microscope has played a major role in research designed to investigate the development of the virus in wart tissue. Almeida et al. (19) found that the virus is formed in association with the nuclei of cells of the stratum spinosum. Virus particles gradually spread throughout the nuclei of the cells in the stratum granulosum and persist as

close-packed aggregates embedded in the substance of the stratum corneum. This correlates to the development of basophilic intranuclear inclusions seen with the light microscope.

Electron microscopy studies also showed that the eosinophilic intranuclear inclusions described by Strauss et al. (20) were the product of abnormal keratinization that occurs in wart cells and were in no way related to the virus. The observations of Lipschutz in 1924, Blank et al. (21) and Arwyn (22) all substantiated these results.

Almeida et al. (19) reported that virus particles could only be seen in the cytoplasm after they were released from the nucleus by disruption of the nuclear membrane. The sequence of development of the human wart virus was similar to the development of Shope papilloma virus in rabbit papillomas. Further, no specific particles could be detected in proliferating cells that were undergoing degeneration and necrosis. It is not known whether the virus was present in the proliferating cells in some form that could not be recognized in the electron microscope or whether the action of the virus in exciting proliferation was an indirect one.

Bloch and Goldman (23) found cells infected with the human wart virus contained a greater amount of deoxyribonucleic acid (DNA) than normal growing cells. The marked intranuclear synthesis of DNA was found to begin early in the course of infection of cells of lower stratum malpighii. In the early phase of infection, an acidophilic intranuclear inclusion

body was recognizable. The inclusion body and nucleus then enlarged and was followed by progressive disorganization of the nuclear structure. The inclusion body became basophilic and stained with the Feulgen stain. Finally the nucleus disappeared leaving the inclusion body in the cell remnant. At a relatively late stage, all the cellular DNA was relocated in the inclusion body and did not significantly increase in amount thereafter.

Williams et al. (24) undertook studies designed to characterize the wart virus morphologically. The nuclei of some cells in the granular layer contained uniform particles of mean diameter 46 mu. Most of the particles consisted of dense core of diameter 34 mu surrounded by a less dense layer of width about 6 mu. In some, the central region was less dense than the periphery. At no time were virus particles seen in the cytoplasm of any of the cells. The shell capsid of the particle was found to consist of 42 capsomeres arranged in a 5:3:2 axial symmetry. The number and arrangement of the capsomeres on the wart virus was established to be the same as that found on the polyoma virus (24). It is of interest that the wart virus which causes benign tumors in man is structurally similar to the polyoma virus which produces a wide spectrum of malignant tumors in several species of animals.

Williams (25) used the acridine orange technique to demonstrate DNA and ribonucleic acid (RNA) in verruca vulgaris. The DNA linked to viral protein was resistant to deoxyribonuclease (DNAase) whereas DNA in normal cells was readily

degraded. In the human wart the epidermal cells containing DNAase resistant nuclei appeared to be situated in areas in which virus particles were found. This led to the conclusion that the DNA in the intranuclear inclusion bodies of human epidermal cells infected with the wart virus is a nucleo-protein. Magis (26) in investigations carried out in our laboratory, infected human skin cell cultures (AU) with verruca virus. Her results showed that within 24 to 48 hours AU cells infected with verruca virus contained intranuclear inclusions of DNA. The inclusions resisted removal by DNAase unless they were first digested with pepsin. At no time were such inclusions susceptible to ribonuclease digestion.

Russell (27) in our laboratory found complement fixing antibodies specific for the wart agent in patients' sera. Under these circumstances the wart agent and antibodies appeared in the same individual. Almeida and Goffe (28) detected antibody to human wart virus by immune electron microscopy or agar-gel precipitin tests using antigen extracted from warts. Antibody was detected in about half a series of 42 patients with warts.

Hayashi (16) could propagate the wart agent only by cell to cell transfer. Dilution of sera or globulin did not prevent infection by the wart virus indicating that large amounts of antibodies were necessary to prevent infection of normal cells by the wart agent. Attempts to establish the agent in monkeys, rabbits, hamsters, mice, and chicken were not successful. By electron microscopy spherical particles

were observed in extracts from wart tissue and infected cultures which were not present in normal skin cells. The size and range of these particles varied from 50 to 75 mu. The particles were not uniform and no particles of any one size were predominant.

Lyell and Miles (29) found eosinophilic, intracytoplasmic and intranuclear inclusion bodies in some warts. They found both type 1 wart and type 2 wart and suggested the name "Verruca Vulgaris" be confined to type 2 warts and that type 1 be renamed "Myrmecia."

Crawford and Crawford (30) studied the nucleic acids of polyoma, Shope rabbit papilloma, bovine papilloma, canine papilloma and human papilloma viruses, with electron microscopy and analytical centrifugation. The polyoma virus differed in size and nucleic acid properties from the papilloma viruses. The four papilloma viruses were similar to each other in many respects but differed significantly in the base composition of their nucleic acids as indicated by different guanine-cytosine ratios.

By electron microscopy Noyes (31) studied fractions of the human wart virus from bands obtained by sucrose density gradient centrifugation. Material obtained from band 3 consisted of intact particles with well preserved structure. The hollow tubular capsomeres that studded the surface of the particles measured about 80Å in diameter and projected outward 50-60Å. In addition, bands 3 and 4 contained some bizarre, elongated, enlarged dumb bell forms whose surface

was composed of capsomeres typical of those covering the particulate form of the wart virus. It was suggested that the small ringlike structures were developmental forms of the human wart virus.

Noyes (32) studied the cytopathic effect of the virus on primary human embryonic skin and muscle cell cultures. Approximately 10^7 to 10^8 intact wart virus particles were required to initiate the cellular change when added to a cell monolayer in a Leighton tube.

Spontaneous regression of naturally occuring human warts usually occurs at puberty (4). It has not been determined whether this is due to antibody formation or other causes such as local tissue resistance. Clarke (33) performed experiments to explore the often-quoted theory of charming warts away. He found no real evidence that magic or suggestion has any effect on warts. He speculated that dramatic cures sometimes reported were probably the result of the magic ritual coinciding with the end of the warts natural life.

The Mitochondria

The generic denomination chondriome embraces all the granular (mitochondria), filamentous or rodshaped (chondrioconts), cytoplasmic bodies that are found in all animal cells, both vertebrate and invertebrate, and in all plant cells. According to Cowdry (34) the terminology applied to mitochondria is rather complicated. At least 50 different names have been used for this organelle. In the Anglo-Saxon

countries the name mitochondria is used, both for the entire group of organelles, chondriosome to each one of them and the names mitochondria and chondriocont to the granular and filamentous forms respectively (35). Lewis and Lewis (36) reported that the mitochondria were also called plastosomen, plasmafaden, plasmakoxen, paramiton, microsomen granules and filaments.

A mitochondrion may be morphologically defined as a cell organelle bounded by a membrane within which are membranous structures (cristae) either villous or vesicular, a ground substance or granular matrix and occasionally, dense granulations. Functionally, mitochondria may be defined as biochemical machines which act as the power-house of a cell (37). They are characterized by a series of physicochemical reactions and staining properties. Among the more important properties are their visibility in vitro, their supravital coloration with stains such as Janus green B, the necessity for special techniques for fixation for their preservation, their lipoprotein composition, their specific ultrastructure and their high concentration of certain enzymes (35).

In 1924 Cowdry (34) reported that mitochondrial changes may be qualitative, quantitative and/or topographical. Owing to the small size of the mitochondria (width 0.2u to 0.5u; length 0.3u to 0.5u) and the limit of the resolution power of the light microscope, qualitative changes can only be detected in optical microscopes by a few criteria. With the advent of the electron microscope, it has become increasingly

easier to observe the mitochondria and mitochondrial changes can be defined in a more diversified and reliable sense.

The mitochondria are ubiquitous in living cells and after some experience they can easily be recognized microscopically. The examination of mitochondria in living cells offers certain difficulties owing to their low refractive index. They are clearly visible with dark field illumination and phase contrast. Vital examination is extremely important in the study of the variations of the mitochondria with time and under the action of different stimuli. The mitochondria are never as conspicuous as the nucleus. They vary greatly in size, shape and position (34, 36, 38). In the living cell, these bodies are continuously changing size, shape and position. Lewis and Lewis (36) observed as many as 20 different shapes exhibited by a single mitochondrion within a period of 15 minutes. Qualitative changes (39) not only involve variations in size and shape of the whole mitochondrion but also its ultrastructure.

Roberts et al., (35) state that the mitochondria have two different types of motion, one of agitation and the other of displacement from one part of the cell to another. Studies of cultured cells by means of moving pictures taken with the phase microscope show that mitochondrial movements are due to cytoplasmic currents. The mitochondria move constantly during interphase but such movement becomes diminished during cell division. Frederick (40) using phase contrast microscopy and microcinematography in the in vitro study of cultures of

fibroblasts, was able to accurately follow the movement of the mitochondria in the resting as well as in the dividing cells. During the displacement, the mitochondria may attach to a certain site on the nuclear membrane.

Mitochondria filaments fragment into granules and these are in turn united together (36). It has also been observed that mitochondria increase in size in hypotonic solutions and diminish in hypertonic solutions. Zollinger (41), Harman (42), Recknagel and Malamed (43) and Claude (44) observed that isolated mitochondria behave as osmometers changing in size with the tonicity of the medium. Their swelling serves as an early sign of cell damage. Rouiller (39) believes that while this explanation is correct in some cases, it cannot become a general rule.

The mitochondria are very labile structures and can be altered by the action of various agents. Lewis and Lewis (36) found that alkalis, ammonia gas and sodium hydroxide cause the mitochondria to swell without any sign of viscosity. Chloroform, xylol, ether and acetic acid dissolve the mitochondria. Janus green B unless used in low concentrations of 1:18,000--1:200,000 is toxic to the cells and causes the mitochondria to divide into granules. These results led Lewis and Lewis to suggest that the mitochondria were concerned with the physiology of the cell and were probably involved in respiration. This suggestion has been confirmed by numerous cytologists and biochemists.

Maley and Johnson (80) found that the swelling of mitochondria is quickened and/or accentuated by pH rise or addition to the media of various substances such as calcium, potassium, phosphates and succinates. Swelling of mitochondria can be slowed by adding adenosine triphosphate (ATP), citrate, magnesium, fluoride and dinitrophenol. Thus, the osmotic phenomenon cannot wholly explain the swelling of mitochondria. Rouiller (39) suggested that mitochondria swell when they lose some ATP as the latter preserve the mitochondrial structures by fixation of the calcium ion.

There are many other mitochondrial changes, most of which are irreversible. Such changes affect: 1. The mitochondrial membrane that becomes single or is disrupted. 2. The cristae, which through vesiculation break up into granulations and disappear. 3. The matrix which assumes a roughly granular, filamentous appearance or else is converted into osmiophilic masses.

Vital and supravital staining is facilitated by coloration with a dilute solution of Janus green B. In this stain the mitochondria become greenish blue in color. Chambers (45) studied this reaction and concluded that it was not due to a chemical combination. He also found that Janus green B had a coagulating effect. He suggested that the stain should not be used as a sole means of identifying mitochondria. However, Lewis and Lewis (36) using Janus green B studied the mitochondria in living cells and found that characteristic mitochondrial shapes were preserved, but that the Janus green B became

toxic to the cells after a period of 90 minutes.

Lazarow and Cooperstein (46) studied the enzymatic basis for the Janus green B staining reaction and found that the reaction was oxygen dependent, that the dye would be reduced by the lactic dehydrogenase enzyme system. The mitochondria take on a greenish blue color which is later reduced to red and finally decolorization occurs. Mitochondria can also be colored in cell culture with methylene blue but this coloration was found to be decolorized by bright illumination (36). The coloration with stains, such as cresyl violet and toluidene blue, may be related to the oxidation-reduction potential of the cell.

It has long been known that mitochondria are labile structures and disintegrate when exposed to fixatives (34, 35, 36). For this reason, all the methods used for their fixation are based on the stabilization of the structures by the prolonged action of oxidizing agents such as osmic acid and potassium bichromate.

Lewis and Lewis (36) found that the mitochondria exhibit extraordinary diversity of form often in the same preparation, even in adjoining cells of the same type. The different mitochondrial shapes may be localized in different parts of the cytoplasm or may be mixed together. Differences between cells of the same type are usually observed during the various stages of mitosis. At one stage the cells may be observed to have similarly shaped mitochondria while in another part practically all the cells may have quite differently shaped

mitochondria. In one preparation all gradations in size and shape may be found. Lewis and Lewis (36) morphologically classified the mitochondria as small granules, dumb-bell shaped granules, spindle-shaped granules, rods, threads, loops, rings and networks. Robertis et al., (35) contend that the shape of the mitochondria, while variable, is basically filamentous or granular. In some cells, especially during functional stages, other forms than the filamentous or granular forms may be seen. Thus, a chondriocont swells at one end to assume a club form, or hollows out at one end to take the form of a tennis racket. The mitochondria at other times may become vesicular in appearance with a central clear zone.

The size of mitochondria is also variable. In most cells the rod-shaped mitochondria are 0.5u to 1u in diameter and of variable length, reaching a maximum of 7u. The size and shape of mitochondria depend also on the pH of the medium. In acid pH they become vesicular. Herring (47) examined fixed and stained preparations of mitochondria and reported that they seem to undergo binary fission. He found that an increase in size of mitochondria could result from a coalescence of two or more mitochondria.

The distribution of mitochondria is in general uniform but there are many exceptions to this rule (35, 36, 39, 47, 48). In some cases they may be accumulated preferentially about the nucleus or in peripheral cytoplasm. During cellular division the mitochondria tend to aggregate about the spindle

and upon division of the cell are distributed in approximately equal quantity among the daughter cells. The distribution of mitochondria within the cytoplasm should be considered in relation to their function as energy suppliers. In some cells they move slowly but in others they have a permanent location near the region of the cell presumably supplied with energy. In some cells the mitochondria provide a definite orientation. In the cylindrical epithelial cells they are generally oriented in the basoapical direction parallel to the main axis (35). Pollister (49) suggested that the orientation mitochondria assume, may depend upon the direction of the currents of diffusion within the cells and would be intimately linked to the submicroscopic structure of the ground cytoplasm.

The mitochondrial content varies with the cell type and functional stage of the cell. In the mouse liver, it has been estimated that the mitochondria account for 30 to 35 per cent of the total nitrogen. In the kidney it represents 17 per cent of the dry weight and 20 per cent of the total nitrogen of the whole tissue (35). On the other hand, in lymphoid tissue values are much lower (50, 51). In mouse liver homogenates about 8.7×10^{10} mitochondria per gram of fresh tissue have been counted (52). In a normal liver cell there are approximately 2500 mitochondria. However, cancer cells contain fewer mitochondria than normal cells. In tumor cells, an average of 786 ± 222 mitochondria per cell were found (53, 54). Howatson and Ham (55) found that in Novikoff's hepatoma there were fewer mitochondria than in normal cells. Each cell

contained an average of 600 mitochondria.

Allard et al., (54) suggested that the small number of mitochondria in tumor cells could be related to scarcity of organized ergastroplasmic structures. The intimate relationship between mitochondria and the ergastroplasm is striking and suggests some sort of dependence of one upon the other. Accordingly a reason for the relative scarcity of mitochondria in malignant cells may be that they are dependent on the coexistence of organized ergastroplasmic structures and there are relatively few of these in tumor cells.

De Robertis et al., (35) attributed the fewer mitochondria in tumor cells to the fact that there is an increase of anaerobic glycolysis with decrease in oxidations in cancer. Junqueira (56) found a direct correlation between number of mitochondria and secretory activity in salivary gland cells.

The advances made in the last twenty years in understanding the mitochondria have been due mainly to refined biochemical techniques, electron microscopy, phase contrast associated with or without microcinematography and differential centrifugation. Such methods have: 1. Made it possible to probe deeper into the ultrastructural complexity of the mitochondrion. 2. The biochemical nature, particularly its enzyme content and the role it plays in cellular metabolism. 3. Its mobility within the cytoplasm. 4. Its share in the formation of various cytoplasmic components. Ultrastructural morphological changes involve biochemical variations. Thus, there is a correlation between morphology and function.

Electron microscopy makes the study of quantitative changes more reliable since through pictures of the mitochondrion it precludes any confusion with other cytoplasmic organelles.

The concept of quantity does not only apply to a change in number of mitochondria but also to its components (increase or decrease in cristae) and chemical composition. Phase microscopy and microcinematography have made it possible to study mitochondrial motion and variations in cell shape in relation to physiological and pathological state of the cells.

Claude and Fullam (57) studied the morphology of isolated mitochondria using the electron microscope. The mitochondria that they studied were obtained from a lymphosarcoma of the rat and were isolated by differential centrifugation. In purified form the mitochondria appeared as spherical bodies (0.6 - 1.3u diameter). Also small elements 80-100u in diameter could be distinguished within the body of certain mitochondria. The isolated mitochondria retained their individuality outside their normal cytoplasmic environment. They persisted as discrete bodies in aqueous media as long as the proper tonicity of the media was maintained.

Most of the knowledge related to mitochondrial ultrastructure has been contributed by Palade (58, 59, 60) and Sjostrand et al., (61, 62, 63). In 1952 Palade demonstrated that mitochondria have a limiting membrane, a system of ridges or cristae in 3 layers protruding from the inside surface of the limiting membrane and a matrix. In 1953 Sjostrand (61), Sjostrand and Rhodin (64) specified that mitochondria are

bounded by double outer continuous membrane within which are inner mitochondrial membranes that form septa which spread across practically the whole membrane width. Contrary to Palade's interpretation, Sjostrand and his co-workers believe that there is a mere topographical connection between the outer and inner mitochondrial membranes. Palade (59) stated that the outer membrane is double and the cristae are folds of the inner membrane layer. This general pattern of organization was found in cells of vertebrates, invertebrates, protozoa and plants.

Elkholm and Sjostrand (65) stated that cristae were an integral part of the inner membrane. From numerous works (63, 66, 67, 68) it may be concluded that a mitochondrion is: 1. Bounded by a continuous membrane consisting of two dense layers divided by a less dense outer membrane. The surface membrane is to be considered as the limiting membrane while the cristae are connected to the inner membrane (59, 66). 2. Inside the mitochondrion is a laminated system, usually at right angles to the main axis of the cell organelle and termed "cristae mitochondriales" and "inner membranes" by Palade and Sjostrand respectively. 3. The matrix contains opaque granules of various sizes. 4. The above general pattern has been found in all cells studied, animal or vegetable.

Although all mitochondria exhibit the same general pattern of organization, ultrastructurally they vary according to species, tissues and organs. Cristae vary in size,

shape, numbers and arrangement within the cell organelle. They are not stable structures and are capable of changing their morphology (39). Differences in number, arrangement and shape of cristae must serve a useful purpose since the membrane including cristae serve as carriers of a large number of enzymes involved in complex biochemical reactions (69). Palade (58) noted a correlation between abundance of cristae and the quantity of enzymes.

Chemically, mitochondria consist mainly of enzymes and lipids (70). Schneider (50) noted the heavy phospholipid content of mitochondria. He assumed that its main purpose was that of a structural component. Sjostrand (61, 63) admitted that the double membranes were protein layers while Dalton and Felix (67) conjectured that the osmiophilic state of the membranes was due to a component lipoidal in nature. Differential centrifugation (50) makes it possible to isolate mitochondria from a broken cell suspension. The homogenate may be divided into four fractions: nuclear, mitochondrial, microsomal and supernatant fluid or soluble fraction. Biochemical analysis of each fraction defines more accurately the chemical composition and the function of each cell constituent. The most striking property of the mitochondria is their enzyme system related to the respiratory activity of the cell. It is in the mitochondria that synthesis of ATP takes place. Owing to its high energy phosphate bonds, the ATP holds a prominent role in cellular metabolism as a source of energy for synthetic reactions.

Mitochondrial alterations in cancer cells are common but are neither constant nor specific (71). Howatson and Ham (55) found that in Novikoff's hepatoma, at pH 7.3, the diameter of the mitochondria was 0.24u to 1.2u while in normal cells it was 0.35u to 0.74u. Elongated forms of mitochondria present in normal cells were absent in tumor cells. The cristae in normal cells were prominent but not as prominent as in tumor cells. The transverse membranes in the mitochondria of tumor cells appeared to branch and anastomose more than those of the mitochondria of normal liver cells. Degenerating mitochondria were larger, had breaks in limiting membranes and revealed only remnants of transverse membranes. The inside of degenerating mitochondria showed a great deal of granular material arranged in irregular clumps suggesting increase in mitochondrial substance.

Increase in mitochondria in Rous Sarcoma was reported by Bernhard et al., (72) and in bone tumor giant cells by Oberling (71). Howatson and Ham (55) reported a decrease in size of mitochondria in Novikoff's hepatoma. Fawcett (73) observed the same in Lucke's renal adenocarcinoma. Mannweiller and Bernhard (74) also observed the same in hamster kidney experimental tumor. Mitochondrial degeneration was demonstrated in Novikoff's hepatoma by Howatson and Ham (55), in the hepatoma of man by Oberling (71) and in chorioallantoic membranes infected with fowl pox, vaccinial viruses and herpes simplex virus by Morgan et al., (75, 76). Dmochowski (77) reported that there may be a decrease in the number of cristae

as in mouse mammary cancer or there may be an increase in the number of cristae as in Novikoff's hepatoma (55).

Benedetti et al., (73) reported that there is an intramitochondrial localization of virus-like particles in avian erythroblastosis. Ackermann and Kurtz (79) using centrifugation techniques for separation of structure units of cells of liver tissue found 16 per cent of the herpes virus bound to the mitochondria by an intimate attachment. Further evidence was obtained that the mitochondria underwent selective deterioration when infected with the virus. The concept was advanced that the organelles function in the development of the virus. Changes in enzymatic activities associated with mitochondria were observed after infection with herpes simplex virus. This observation led Ackermann and Kurtz to suggest that the mitochondria were a site of herpes simplex virus synthesis.

MATERIALS AND METHODS

All reagents were made with two cycle, all glass distilled water. Solutions used as growth and maintenance media were made with two cycle, all glass distilled water. At times the composition of the media were altered in order to meet the specific requirements of each experiment.

Reagents

Hanks' balanced salt solution (82).

Solution 1.---Contained 0.7 gm calcium chloride in 100 ml double distilled water.

Solution 2.---Contained the following reagents in 450 ml of double distilled water.

	Grams		Gram
Glucose	5	KH_2PO_4	0.3
NaCl	40	$Na_2HPO_4 \cdot 2H_2O$	0.3
KCl	2	Phenol Red	0.1
$MgSO_4 \cdot 7H_2O$	1 gram		

The two solutions were autoclaved separately at 10 lbs pressure (115°C) for 10 minutes, cooled to room temperature and then combined. The pH was adjusted to 7.0-7.2 with sterile solution of 7% $NaHCO_3$.

Basal Medium Eagle (83).

10X Stock Solution

NaCl	34 grams
KCl	2.0 grams
$NaH_2PO_4 \cdot H_2O$	0.70 gram

(continued on next page)

10X Stock Solution (continued)

$CaCl_2$	1.0 gram
$MgSO_4 \cdot 7H_2O$	1.0 gram
Glucose	5.0 grams
Phenol Red	0.1 gram

The reagents were dissolved in 500 ml of double distilled water, sterilized by Seitz filtration and the pH adjusted to 7.0-7.2 with sterile 7% $NaHCO_3$. One ml of each mixture of vitamins and amino acids (Microbiological Associates, Inc., Bethesda, Maryland.) were added just before use. The composition of the vitamin and amino acid mixtures, each dissolved in a liter of double distilled water was as follows:

Vitamin mixture		Amino acid mixture	
Biotin	0.1 gram	Arginine	2.1 grams
Choline	0.1 gram	Cystine	1.2 grams
Folic acid	0.1 gram	Histidine	0.8 gram
Nicotinamide	0.1 gram	Isoleucine	2.6 grams
Pantothenic acid	0.1 gram	Leucine	2.6 grams
Pyridoxal	0.1 gram	Lysine	2.6 grams
Thiamin	0.1 gram	Methionine	0.8 gram
Riboflavin	0.01 gram	Phenylalanine	1.6 grams
i-Inositol $\cdot 2H_2O$	0.18 gram	Threonine	2.4 grams
		Tryptophan	0.4 gram
		Tyrosine	1.8 grams
		Valine	2.4 grams
		Glutamine	30.0 grams

Yeast Extract Medium, YEM (84)

This medium consisted of 0.1% yeast extract and 0.35% glucose in Hanks' balanced salt solution.

Antibiotic Solution

Penicillin (100 units) and dihydrostreptomycin (100 ug) per ml were added to all nutrient media. Tissue samples were treated by placing into a solution containing 500 units of penicillin and 500 ug of dihydrostreptomycin. Occasionally, oxytetracycline (Terramycin) was added to the media, 5 ug per ml or less.

Trypsin

Trypsin (1:250, Difco) was prepared by dissolving 0.25 gm in 100 ml calcium free Hanks' balanced salt solution.

The trypsin solution was sterilized by Seitz filtration. The pH was adjusted to 7.0--7.2 by addition of sterile 7% $NaHCO_3$. Cell monolayers were exposed to trypsin for periods of ½ to 1 minute. The trypsin was then removed with a pipette.

The AU Cell Line

The history of the AU cell line used for this study was adequately described by Wheeler et al., (81). Originally the epidermal cells were obtained from a 17 year old boy (AU). The skin tissue was trypsinized and placed in growth medium which consisted of 40% pooled human serum, 5% chick embryo extract and 55% Hanks' balanced salt solution. One hundred units of penicillin and 100 ug of dihydrostreptomycin per ml were used in all media.

The AU cells were grown in 150 ml capacity milk dilution bottles or Leighton culture tubes. The cells adhered to the glass surface and produced a confluent sheet of epithelial-like cells in from two to five days.

When originally received in this laboratory the cells required human serum for growth. The cells were adapted to growth in 20% calf serum. After a sheet of cells developed, the cultures were transferred by treating the cell monolayer with 0.25% trypsin (1:250, Difco) and transferring the cells to two bottles. The serum concentration in the medium was reduced to 2% to maintain the monolayer.

The cell line was not homogeneous. Most and sometimes all the cells in a monolayer were epithelioid (Fig. 1) but at other times many of the cells had fibroblastic-like appearance.

Growth of AU Cells in Leighton Tubes

Leighton tubes containing coverlips were seeded with one ml of growth medium containing approximately 500,000 cells and were stationary incubated at 37°C. After a confluent monolayer was obtained small wart fragments were added. Growth medium consisted of YEM supplemented with 20% heat inactivated calf serum and 0.5% lactalbumin hydrolysate, (LAH) (Difco).

After a confluent monolayer of cells was formed and before it was infected with the wart fragments, growth medium was replaced by maintenance medium which consisted of YEM supplemented with 2% inactivated calf serum.

Samples of cells were removed at specific intervals and either vitally stained with Janus green B or else fixed for staining with acid fuchsin or hematoxylin.

The Wart Tissues

The wart tissues were obtained from a dermatologist's office in Detroit, Michigan. They were removed by total enucleation (85). The tissues were immediately placed in vials containing sterile 50% phosphate buffered glycerine. Upon arrival to the laboratory they were kept refrigerated at 4°C. Before use they were washed with Hanks' balanced salt solution and then treated with heavy doses of the antibiotic solution for varying periods of 24 to 48 hours. They were minced with scissors and/or scalpels and the small fragments of wart tissues were added to AU cell monolayers in tissue culture glass bottles or Leighton tubes. In some experiments the wart tissue fragments were first heated to 56°C for 30 minutes, cooled to room temperature and then added to AU monolayers.

Fresh wart tissues were removed from individuals in the laboratory. They were treated immediately with the antibiotic solution and held at 4°C.

Cell free extracts were obtained by grinding the wart tissue in a mortar and pestle. Hanks' balanced salt solution containing penicillin and streptomycin was used as diluent. A 5% suspension of wart material was centrifuged in a horizontal position at 795 x g for 10 minutes to remove the cell

debris and unbroken cells. One ml of the supernatant fluid was added to AU cells.

Sterility Tests

Sterility tests for bacterial contamination were made of all culture materials in brain heart infusion broth (Difco). They were considered free of viable bacteria if there was no turbidity after 72 hr incubation at 37°C.

Some cultures were tested for the presence of pleuropneumonia-like organisms by seeding cells and samples of cell cultures into pleuropneumonia-like organisms medium (Difco).

Fractionation of AU Cells

The procedure used for fractionation was that of Bensley and Hoerr (86) with minor modification borrowed mainly from the procedure of Hogeboom et al., (87).

After cytopathogenic effect (CPE) was detected, infected cell monolayers and uninfected cultures were mechanically loosened from the glass wall of the bottle, removed, and suspended in 3 to 5 ml of Hanks' balanced salt solution. The infected and uninfected cell suspensions were pipetted into each of two weighed culture tubes. The tubes, were centrifuged for 5 minutes in the horizontal position at 795 x g and the solution was removed with a pipette. The wet packed cells were weighed using a Christian Becker analytical balance. Each cell sample was then suspended in 2 ml of 0.88 M sucrose solution (Merck reagent grade) at

pH 7.0--7.2. The infected cells and control cells were placed into two different all-glass tissue microgrinders. Grinders were cooled by placing the tubes in ice-baths and grinding produced a homogenized paste in 1 to 2 minutes of grinding. Care was taken to keep the specimens cool (0--4°C) in the subsequent steps of separation. Each homogenate was diluted to a total volume of 15 ml.

Separation of Cell Components

The unbroken cells, nuclei and cell debris were removed from the suspension by centrifugation. The homogenate was centrifuged at 795 x g for three minutes at 4°C (International multispeed centrifuge). The supernatant fluid was removed from the sediment and the fluid was centrifuged again for three minutes as above. A third centrifugation was done resulting in slightly opalescent suspension. The sediments were discarded. One ml of 0.88 M sucrose was placed in a conical tube and 5 ml of the suspension was carefully layered over the sucrose solution forming a two-phase system. The mixture was undisturbed for 10 minutes at 4°C. The mixture was centrifuged (795 x g) in a horizontal position for 4 minutes at 4°C and then the upper phase of the mixture was carefully removed. Examination of the sediment after this treatment, by staining with Janus green B showed that the method removed only occasional mitochondria. Examination of the upper phase supernatant fluid contained mainly free mitochondria and some cell masses.

Isolation of Mitochondria

The suspension containing free mitochondria plus other elements derived from the cytoplasm of AU cells was centrifuged for 30 minutes at 17,400 x g to sediment the mitochondria. The firmly packed, opaque sediment of mitochondria was suspended in 0.88 M sucrose solution. In order to free the mitochondria preparation from the soluble substances in the original homogenate the mitochondria were resedimented at 17,400 x g and resuspended again in 0.88 M sucrose. The supernatant fluid was examined for presence of mitochondria. None was found. The final sediment was yellowish in color and after dilution with sucrose solution constituted the mitochondrial suspension.

Mitochondria Counts

Mitochondria counts were made using the method of Harman (88) with minor modifications. A known volume of suitably diluted mitochondria preparation was spread over a measured area on a microscope slide. The smear was fixed in 4% formalin or exposed to osmium tetroxide fumes (2% osmic acid) at room temperature and stained with acid fuchsin. The stained mitochondria were counted with a Bausch and Lomb (Apochromatic) microscope fitted with 10X ocular and 90X (oil) objectives. For each dilution, at least three slides were prepared and in each slide twenty fields were counted. The total number of mitochondria per gram of wet AU cells was calculated from the known volume of suspension, the area occupied, the total number of fields per unit area and the weight of the cells.

Photography

Microphotographs were taken with a Bausch and Lomb camera using Kodak Panatomic-X sheet film and Panatomic-3X sheet film. The film was developed in DK50 or Microdol-X (Kodak) and fixed in sodium thiosulfate (hypo).

I. Growth of Verruca Virus in AU Cells at 37°C

As found by Hayashi (16) and Magis (26) AU cell cultures inoculated with small fragments of recently removed warts were infected with the wart virus. The evidence for this infection was a progressive CPE. Most of the cells in the monolayers became round. The rest of the cells became angular and occasionally formed microvilli. The cytoplasm of the cells showed increasing granulation. As degeneration continued cells began to aggregate and detached from the walls of the bottles or Leighton tubes, usually by five days post inoculation. Uninfected cultures did not show such an effect. Cultures which were inoculated with heated wart fragments did not show any CPE.

Heavy suspensions of infected cells from these cultures were used for serial passage of the wart agent. Infected monolayers again demonstrated the cytopathogenic effect as described above. Degeneration was evident 7 to 10 days post inoculation. The time required for degeneration to occur varied between 5 to 10 days. Uninfected cultures did not show any CPE.

The above cultures were grown on YEM medium containing 20% calf serum and maintained on 2% serum. When AU cell cultures were grown on Eagles' basal medium plus serum, 1 to 3 additional days of incubation were required before CPE took place.

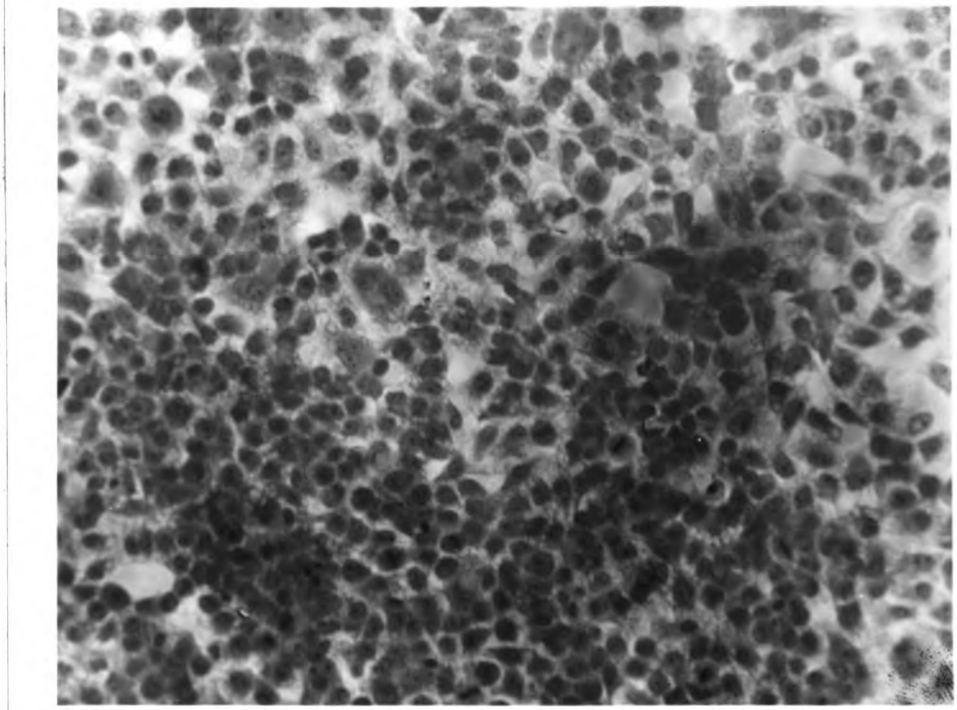

Fig. 1. Normal AU cells stained with hematoxylin and eosin. Magnification X240.

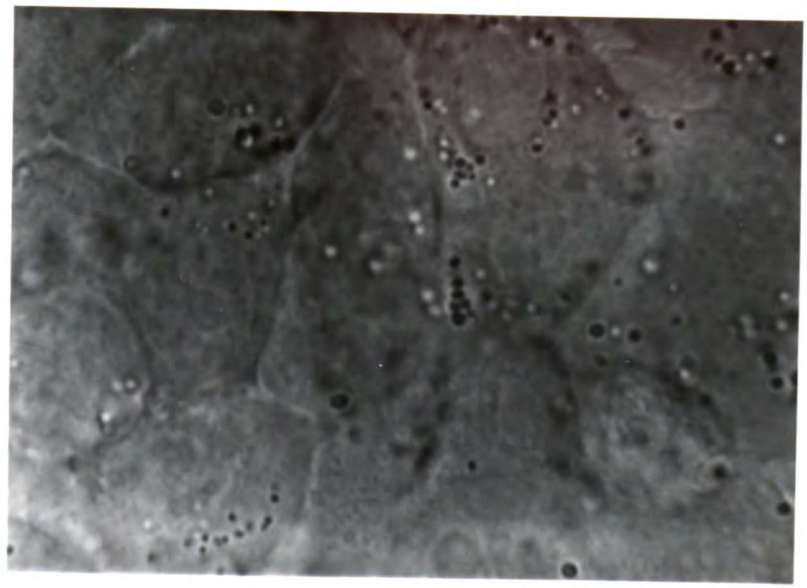

Fig. 2. Mitochondria in normal control cells vitally stained with Janus green B. Magnification X1100.

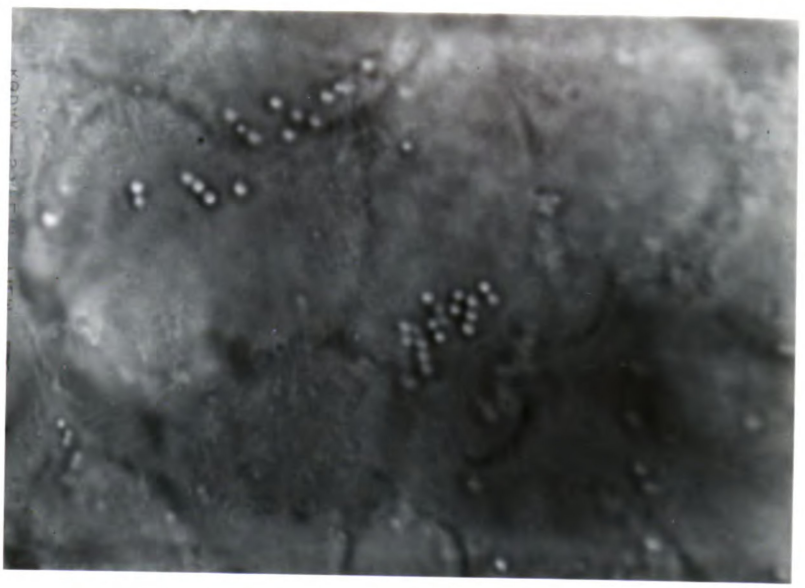

Fig. 3. Mitochondria in infected cells, 5 days post inoculation. Magnification X1100.

Occasionally, areas of "resistant" cells were found in the monolayers. Attempts to grow these cells in large quantities for fractionation were unsuccessful.

II. Cell Fractionation

In preliminary studies it was found that distilled water or isotonic saline solution did not satisfy the cytological criteria of integrity so necessary for the identification of mitochondria. The two media caused extensive swelling of the mitochondria and mitochondria freed in either water or saline solution were spherical. In an isotonic solution, the mitochondria were refractive whereas in water they were larger and pale in color. In both media they stained weakly or not at all with Janus green B. Mitochondria isolated in isotonic saline solution tended to agglutinate and resulted in a prohibitive loss when attempts were made to isolate the mitochondria free from nuclei and unbroken cells.

A 0.88 M sucrose solution was satisfactory for the centrifugal fractionation of AU cells. The mitochondria varied greatly in length when isolated in 0.88 M sucrose solution, the majority of them being 1-4u in length and the remainder either granules or slightly elongated rods and filaments. They were readily stained with a solution of Janus green B in 0.88 M sucrose solution (dye concentration 1:10,000-1:30,000) introduced under the coverslip. The staining reaction was faint when a dye concentration

of 1:40,000 was used. The morphological, cytological characteristics and the ability to stain with Janus green B were the criteria used for identification of mitochondria.

When uninfected cells grown on coverslips in Leighton tubes were stained with Janus green B, it was found that the mitochondria decolonized rapidly upon exposure to bright illumination. In uninfected cells, most of the mitochondria were short rods, others were comma-shaped and the rest were spherical granules (Fig. 2). This was generally true regardless of the medium used for growth. Infected cells stained poorly with Janus green B and decolorized rapidly upon exposure to bright illumination. The mitochondria in infected cells (Fig. 3) became filamentous resulting in a stringy network. This was generally true regardless of the medium used.

Occasionally, unbroken AU cells in the homogenates were stained with Janus green B. In uninfected cells, the intracellular mitochondria were predominantly rod-like. Other mitochondria were comma-shaped and the rest spherical. In infected cells the intracellular mitochondria were mainly filamentous. The morphology of mitochondria isolated from the cells and those seen in the intact cells appeared similar. However, mitochondria isolated from infected cells appeared either elongated or slightly swollen. Unlike some cells (87), the isolated mitochondria from the AU cells did not require a high concentration of dye to be stained suitably.

When 0.83 M sucrose homogenates of AU cells were fixed by addition of an equal volume of 2% aqueous solution of osmium tetroxide and the mixture allowed to stand 9 to 12 hours at 4°C, smears were suitable for staining with standard cytological techniques. However, fixation and smearing distorted some of the elongated mitochondria. The fixed mitochondria were deeply stained by acid fuchsin, hematoxylin and basic dyes such as safranine and crystal violet.

III. Mitochondria Counts in Normal and Infected Cells

When fresh wart fragments were seeded onto monolayers of AU cells there was, in every instance, an increase in number of mitochondria found in infected cells (Table 1). When the original passage was followed by serial passages of the infected cells, there were always more mitochondria found in infected cells than in uninfected cells. The mitochondria count on uninfected cells, original and passages, varied from 4.30×10^8 to 5.51×10^8 with an average of 4.98×10^8 mitochondria. A count of the mitochondria from wart infected cells ranged from 1.65×10^{10} to 2.22×10^{10} giving a 1.99×10^{10} average number of mitochondria per gram of wet AU cells. There was approximately a forty-fold more mitochondria in infected cells than in uninfected cells (Table 1).

Counts of mitochondria from passage to passage varied slightly in both infected and uninfected cultures. There was no noticeable trend apparent of increase or decrease in numbers as passages were continued.

When glycerinated wart fragments were used as infecting material, there were more mitochondria in infected cells than in uninfected cells (Table 2). In uninfected cells the number of mitochondria varied from 4.82×10^8 to 5.77×10^8 per gram of wet AU cells with an average of 5.15×10^8 mitochondria. A count of the mitochondria in cell monolayers seeded with glycerinated wart fragments varied from 1.54×10^{10} to 2.30×10^{10} with an average count of 1.94×10^{10} per gram of wet AU cells.

There was approximately a thirty-eight fold more mitochondria in infected cells than in uninfected cells (Table 2). The mitochondria counts in cells that were infected with glycerinated wart fragments were comparable to those counts that were obtained when fresh wart fragments were used as infecting material (Table 1).

When uninfected cells were grown on a complex and complete medium there was an increase in mitochondria count. Cells grown on Eagles' medium (Table 3) contained more mitochondria than cells grown on an incomplete medium such as YEM (Table 1). The mitochondria count in infected cells, original and passage varied from 1.33×10^{12} to 1.77×10^{12} with an average count of 1.55×10^{12} per gram of wet AU cells. In the case of uninfected cells, the mitochondria count ranged from 1.49×10^9 to 2.88×10^9 with an average count of 2.09×10^9 per gram of wet cells (Table 3). As in Table 1,

there was no noticeable trend of increase or decrease in numbers of mitochondria as passages were continued. It was also found that infected cells had a seven hundred and forty fold increase in number of mitochondria when compared with the number found in uninfected cells. Uninfected cells grown in Eagles' basal medium contained a 4.2 fold increase in number of mitochondria as compared to uninfected cells grown in YEM (Tables 1, 3). Mitochondria counts in infected cells (Table 3) grown in Eagles' medium, while exhibiting some variation, showed no noticeable trend in numbers as passages were continued.

Uninfected cells grown in Eagles' medium (Tables 3, 4) contained more mitochondria per gram of AU cells compared with uninfected cells grown in YEM (Tables 1, 2). The mitochondria count (Table 4) in uninfected cells ranged from 1.27×10^9 to 2.74×10^9 with an average count of 2.25×10^9 per gram of wet AU cells. In the infected cells (Table 4) the mitochondria count varied from 1.20×10^{12} to 1.91×10^{12} with an average count of 1.59×10^{12} per gram of wet AU cells. Infected cells had seven hundred fold more mitochondria than uninfected cells.

The results of mitochondria counts in cells inoculated with heated wart material are summarized in Tables 5 and 6. No separate controls were done for the heated wart material but the controls as done in Tables 1, 2,

3 and 4 were used for comparison. Cells grown in the more complete basal medium (Table 5) contained more mitochondria per gram of cells than cells grown in the incomplete yeast extract medium (Tables 1, 2 and 6). The results in Tables 5 and 6 also indicate that there was no appreciable increase or decrease in mitochondira count in cells that were inoculated with heated wart fragments. The original passage was followed by serial passages of the unheated cells. The results in Tables 5 and 6 indicate that there was no noticeable trend of increase or decrease in numbers of mitochondria as passages were continued.

TABLE 1

Average number of mitochondria per gram of AU cells, uninfected or infected with fresh wart virus.

Experiment	Infecting Material	No. of Dilutions Done	No. of Slides per dilution	No. of Fields Counted per Slide	No. of Mitochondria in Infected AU Cells	No. of Mitochondria in Uninfected* Cells
1	Fresh Wart Fragments	5	3	20	2.17×10^{10}	4.90×10^{8}
2	Infected Cells 1st Passage	5	3	20	1.95×10^{10}	4.30×10^{8}
3	Infected Cells 2nd Passage	5	3	20	1.87×10^{10}	5.32×10^{8}
4	Infected Cells 3rd Passage	5	3	20	1.65×10^{10}	5.21×10^{8}
5	Infected Cells 4th Passage	5	3	20	2.13×10^{10}	5.51×10^{8}
6	Infected Cells 5th Passage	5	3	20	2.22×10^{10}	4.65×10^{8}
			Average Number		1.99×10^{10}	4.98×10^{8}

* Normal uninfected cells were used in the original and subsequent serial passages. Cultures were maintained in yeast extract medium and 2% calf serum.

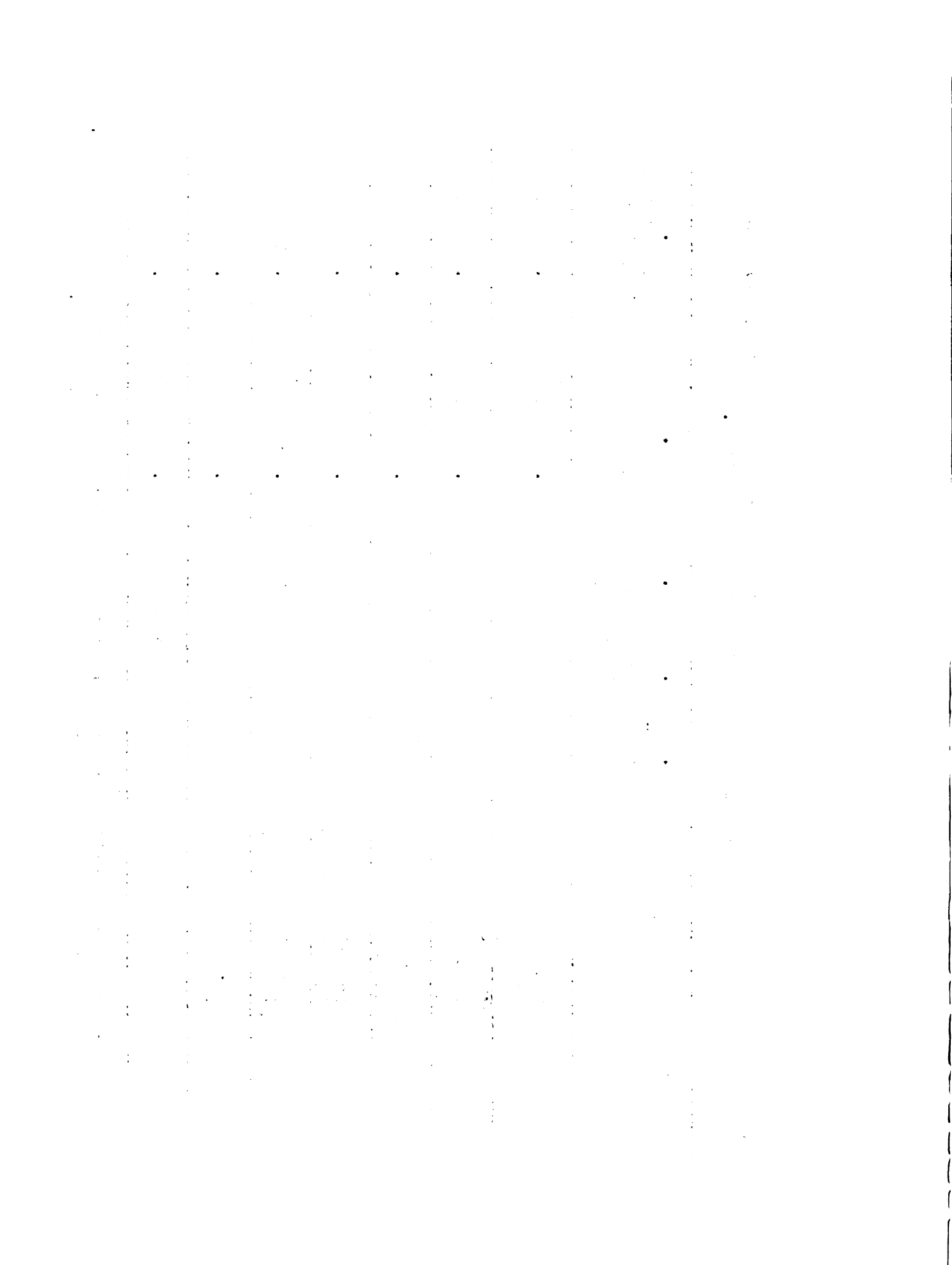

TABLE 2

Average number of mitochondria per gram of AU cells, uninfected or infected with glycerinated wart virus.

Experiment	Infecting Material	No. of Dilutions Done	No. of Slides per Dilution	No. of Fields Counted per Slide	No. of Mitochondria in Infected AU Cells	No. of Mitochondria in Normal *Control Cells
1	Glycerinated Wart Fragments	5	3	20	1.54×10^{10}	4.82×10^{8}
2	Glycerinated Wart Fragments	5	3	20	2.28×10^{10}	5.77×10^{8}
3	Glycerinated Wart Fragments	5	3	20	1.62×10^{10}	4.94×10^{8}
4	Glycerinated Wart Fragments	5	3	20	2.30×10^{10}	5.09×10^{8}
				Average Number	1.94×10^{10}	5.15×10^{8}

* Normal uninfected cells were seeded onto normal AU cell monolayers. Cultures were maintained in yeast extract medium and 2% calf serum.

TABLE 3

Average number of mitochondria per gram of AU cells, uninfected or infected with fresh wart virus.

Experiment	Infecting Material	No. of Dilutions Done	No. of Slides per Dilution	No. of Fields Counted Per Slide	No. of Mitochondria in Infected AU Cells	No. of Mitochondria in Normal * Control Cells
1	Fresh Wart Fragments	5	3	20	1.44×10^{12}	1.82×10^{9}
2	Infected Cells 1st Passage	5	3	20	1.77×10^{12}	1.96×10^{9}
3	Infected Cells 2nd Passage	5	3	20	1.33×10^{12}	2.88×10^{9}
4	Infected Cells 3rd Passage	5	3	20	1.55×10^{12}	2.60×10^{9}
5	Infected Cells 4th Passage	5	3	20	1.69×10^{12}	1.49×10^{9}
6	Infected Cells 5th Passage	5	3	20	1.55×10^{12}	1.77×10^{9}
			Average Number		1.55×10^{12}	2.09×10^{9}

* Normal uninfected cells were used in the original and subsequent serial passages. Cultures were maintained in Eagles' basal medium and 2% calf serum.

TABLE 4

Average number of mitochondria per gram of AU cells, uninfected or infected with glycerinated wart virus.

Experiment	Infecting Material	No. of Dilutions Done	No. of Slides per Dilution	No. of Fields Counted per Slide	No. of Mitochondria in Infected AU Cells	No. of Mitochondria in Normal *Control Cells
1	Glycerinated Wart Fragments	5	3	20	1.84×10^{12}	1.27×10^{9}
2	Glycerinated Wart Fragments	5	3	20	1.91×10^{12}	2.74×10^{9}
3	Glycerinated Wart Fragments	5	3	20	1.41×10^{12}	2.46×10^{9}
4	Glycerinated Wart Fragments	5	3	20	1.20×10^{12}	2.52×10^{9}
				Average Number	1.59×10^{12}	2.25×10^{9}

* Normal uninfected cells were seeded onto normal AU cell monolayers. Cultures were maintained in Eagles' basal medium and 2% calf serum.

TABLE 5

*Average number of mitochondria per gram of AU cells seeded with glycerinated wart material heated to 56°C for ½ hr.

Experiment	Infecting Material	No. of Dilutions Done	No. of Slides per Dilution	No. of Fields Counted per Slide	No. of Mitochondria per Gram of Wet Cells
1	Heated Glycerinated Wart Fragments	5	3	20	2.54×10^9
2	Unheated Cells 1st Passage	5	3	20	1.93×10^9
3	Unheated Cells 2nd Passage	5	3	20	2.07×10^9
4	Unheated Cells 3rd Passage	5	3	20	2.11×10^9
5	Unheated Cells 4th Passage	5	3	20	2.05×10^9
6	Unheated Cells 5th Passage	5	3	20	2.34×10^9
				Average Number	2.17×10^9

*Cells were grown on basal medium (Eagles) and maintained in 2% calf serum.

TABLE 6

*Average number of mitochondria per gram of AU cells seeded with glycerinated wart material heated to 56°C for ½ hr.

Experiment	Infecting Material	No. of Dilutions Done	No. of Slides per Dilution	No. of Fields Counted per Slide	No. of Mitochondria per Gram of Wet Cells
1	Heated Glycerinated Wart	5	3	20	5.13×10^8
2	Infected Cells 1st Passage	5	3	20	5.39×10^8
3	Infected Cells 2nd Passage	5	3	20	4.79×10^8
4	Infected Cells 3rd Passage	5	3	20	4.96×10^8
5	Infected Cells 4th Passage	5	3	20	5.08×10^8
6	Infected Cells 5th Passage	5	3	20	4.87×10^8
				Average Number	5.03×10^8

* Cells were grown on yeast extract medium and maintained with 2% calf serum.

DISCUSSION

I. Mitochondria Counts

Few successful isolations of the wart virus have been accomplished. Although Mendelson and Kligman (15) reported the cultivation of this virus in monkey kidney cell cultures, this has not been confirmed. Their work lacked adequate controls. Hayashi (16) and Magis (26) found that this agent could be serially carried in AU cells. In order to infect cell cultures with the wart virus, wart tissue fragments are required. Cell-free extracts of wart material do not initiate infection.

The close association of the virus with the cell prompted a comparison of the components of the infected cells to uninfected cells. Although infection of AU cells results in degeneration there are many other changes, both physical and chemical, that take place in cells infected with verruca virus. Such changes affect the components of the cells in various ways. Leuchtenberger and Leuchtenberger (89) found normal skin cells in vivo contained a constant content of DNA while skin cells infected with verruca virus had a higher DNA content. The DNA content varied from cell to cell. In other studies, Leuchtenberger and Leuchtenberger (89) found that the polyoma virus which is structurally similar to the verruca virus caused an increase in mitochondria of monkey kidney cell cultures. Our results summarized in Tables 1, 2, 3 and 4 show that when AU cells were infected

with the human wart virus, there was an increase in the number of mitochondria of the cells in every instance.

Magis (26) observed that when AU cells were grown on a rich medium (LAH), evidence of infection (CPE) required an additional one or two days. She grew the cells on an incomplete medium (YEM) plus serum or a more complete medium (LAH) plus serum. This observation prompted an investigation as to what effect the more complete medium had upon the number of mitochondria in infected and uninfected cells. To enhance the possible results, Eagles' medium (a very complete medium) was used to grow the AU cells. The results were then compared to the results obtained from counts of mitochondria obtained from cells grown in an incomplete medium (YEM).

The total number of mitochondria were always increased in cells grown on the complete medium. Both infected cells (Tables 3, 4) and uninfected cells always had more mitochondria than their counterpart (Tables 1, 2).

All control cell cultures consisted of monolayers of AU cells which were seeded with 1 ml of a suspension of uninfected AU cells. No attempt was made to determine the number of viable cells in this inoculum. Since this method was also used to seed infected cultures (except using infected cells), approximately the same number of cells were used as seed for both infected and uninfected cultures. If the normal cells added to monolayers attached to the walls of the bottle and grew, the normal control

bottles should contain more cells and therefore more mitochondria. This was not the case, in fact, in every instance the uninfected cultures had a lower mitochondria count. The infected cell was able to produce more mitochondria per cell than the uninfected. It is possible that some of the infected cells also attached and multiplied, however, wart virus infected cells usually round up and deattach from the vessel wall and do not possess the ability to attach to the walls again.

The possibility that the increase in mitochondria counts in infected cells may have been caused by factor(s) other than the virus in the wart material was considered. To determine whether the increase was actually due to the virus and not other factor(s), some wart material was first brought to 56°C for ½ hr. This procedure inactivated the virus. The heated wart material was then used to inoculate AU cells and mitochondria counts were done. There was no apparent increase or decrease in mitochondria in cells seeded with heated material (Tables 5, 6). The results closely parallel those obtained with uninfected cells seeded with normal cells. It is assumed therefore that the increased mitochondria counts were due to the presence of the virus.

The mitochondria counts from the different homogenates obtained from cells grown under similar conditions varied some. This raised the question whether the variation was caused by an inability to homogenize

cells equally each time or whether the differences represented a true cell variation. No statistical analyses were made. However, in a similar study, Shelton et al. (52) statistically analysed the mitochondria variation in homogenates prepared from C_3H mice livers. The homogenate differences were not due to errors in the technique of homogenization but rather an indication of animal variation.

II. Cell Fractionation

Cell fractionation has been used extensively in the study of cellular components, particularly by Hogeboom et al. (87). One main advantage of this procedure is that cell fractions can be obtained in large amounts for extensive cytological and biochemical study. On the other hand, the possibility that artifacts may be produced in the course of fractionation cannot be discounted. The artifacts may include: 1. The morphological alteration of intracellular components particularly the mitochondria which are very sensitive to extraneous interference, may be so extensive that the mitochondria may not be identifiable; 2. When cell membranes are broken a change in the biochemical properties may result from either adsorption or the loss of soluble substances from the mitochondria. In attempts to characterize biochemically or cytologically the mitochondria in the isolated state, it is important to demonstrate their

integrity as well as the degree of homogeneity of the preparation.

III. Mechanism of Effect of Concentrated Sucrose Solutions

The mechanism by which a hypertonic sucrose solution is capable of preserving the morphological and cytological characteristics is not understood (87). It is believed that the effect is largely one of tonicity. In a series of homogenates prepared in preliminary experiments, the proportion of rodlike forms progressively increased with increasing sucrose concentration. Conversely, when homogenates prepared in 0.88 M sucrose were diluted with distilled water to a final concentration of 0.25 M, the rodlike mitochondria became spherical within 4-6 minutes. The change from rodlike to spherical shape was accelerated in a thin film on the microscopic slide. Mitochondria suspended in the diluted homogenate became spherical at a lower rate than these observed on the microscope slide.

In unbroken uninfected cells the intracellular mitochondria remained predominantly rodlike. The mitochondria in unbroken infected cells were of different shapes, some were rodlike, a few spherical but the bulk of them were filamentous. The cells did not show shrinkage in the hypertonic medium. Brownian movement within the uninfected and infected unbroken cells in the homogenates was greatly reduced. Hogeboom

et al. (87) observed the same phenomenon in rat liver cells and concluded that the cytoplasm had a gel-like consistency. They stated that other factors than the osmotic pressure of 0.88 M sucrose solution may play an important role in maintaining the integrity of mitochondria. The high osmotic pressure in the medium may reflect more than a control of the distribution of water within and outside the mitochondria.

The 0.88 M sucrose solution used has some disadvantages. Because of its high viscosity, high centrifugal forces must be used in centrifugation. Hogeboom et al. (87) believe that the high sucrose concentration may adversely affect the enzymatic properties of mitochondria.

In conclusion, the results obtained in this work were in general agreement with those of other workers. Many viruses alter the morphology of mitochondria of infected cells. The majority of viruses cause an increase in mitochondria of infected cells.

SUMMARY

1. AU cells which were inoculated with fragments of wart tissue became infected and exhibited a progressive cytopathogenic effect. The infected cells became round or angular and occasionally formed microvilli. The cytoplasm of the cells showed increasing granulation.
2. Uninfected cells which were grown on Eagles' medium plus serum contained more mitochondria than normal control cells grown on yeast extract medium and calf serum.
3. Infected test cells grown on Eagles' medium contained more mitochondria than infected cells grown on yeast extract medium.
4. Infected test cells grown on either Eagles' medium or yeast extract medium contained more mitochondria than their respective uninfected cells.
5. Cells which were seeded with heated wart material did not show any increase in mitochondria counts.
6. Mitochondria in infected cells were more filamentous whereas those in uninfected cells were mainly rodlike.

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