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BY

THOMAS KAY SHIRES

Oklahoma City, Oklahoma

1965

AN ANALYTIC EVALUATION OF THE INTRACELLULAR

DISTRIBUTION OF CARCINOGENIC HYDROCARBON

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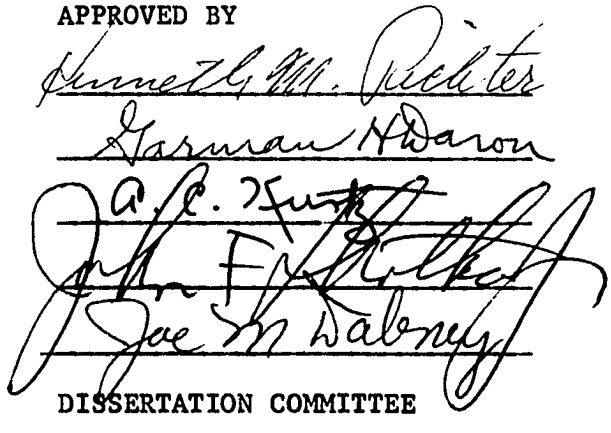

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CHAPTER I

INTRODUCTION

An understanding of the mode of action of the fluorescent, polycyclic carcinogenic hydrocarbons (CH) has been viewed as part of the broad, unanswered, fundamental question concerning the natural etiology of neoplasia (Boyland, 1952; Miller and Miller, 1952, 1959; Haddow, 1958; Heidelberger, 1959; Prehn, 1964). In any attempt to uncover and explain the events linking application of a carcinogen to a living tissue and the appearance of tumors in that tissue, the precise intracellular primary sites of reaction with the hydrocarbon must be established (Miller, 1951; Weist and Heidelberger, 1953a; Haddow, 1958; Heidelberger, 1959; Pitot and Heidelberger, 1963; Boyland, 1964). In a cytomorphologic approach to this problem, a number of u-v-fluorescence microscopists studying a wide range of vertebrate and invertebrate cell types under physiologic condition in vitro have found a pattern of intracellular CH which is common to all nucleated cell types (Richter, 1951, 1952a and b, 1955: Richter and Saini, 1960; Shires, 1962; Jones, 1962; Harvey et al., 1963). This pattern includes relatively high demonstrable accumulations of CH in lipid granules and

elements of the Golgi complex, and no demonstrable uptake of CH by mitochondria, cytoplasmic vacuoles and nuclear structures, such as nucleoli, chromatin and achromatic karyoplasm.

With this pattern of CH within living cells as a basis for comparison, it is notable that cell study using different methods has produced different results: 1) Graffi (1939, 1941), studying living cells exposed to CH via a glycerine-containing medium and observed with u-v-fluorescence microscopy, reported the accumulation of CH by intranuclear structures identified as nucleoli. 2) Ahlström and Berg (1949) and Ahlström (1949), using epidermis fixed in formaldehyde, sectioned on a freezing microtome and observed with u-v-fluorescence microscopy, reported marked uptake of CH by mitochondria. 3) Reports by Calcutt and Payne (1953, 1954) and by Woodhouse (1954, 1955) have indicated significant concentrations of CH in subcellular liver nuclear and mitochondrial fractions using fluorimetric determination. 4) High levels of activity of C^{14}-labeled CH were found in nuclear and mitochondrial subcellular fractions of rat epidermis (Weist and Heidelberger, 1953b and c). 5) Isolation of CH strongly bound to DNA isolated directly from epidermis has indicated to some authors penetration of the nucleus by CH (Weist and Heidelberger, 1953b and c; Heidelberger and Davenport, 1961; Brookes and Lawley, 1964). 6) Polkinka (1958) reported intranuclear fluorescence in formaldehyde-fixed, frozen-sectioned epidermal cells after painting the skin with CH.

All of these reports are in disagreement with the data derived by u-v-fluorescence microscopic examination of living cells under

in vitro conditions (Richter, 1951, 1952a and b, 1955; Richter and Saini, 1960; Shires, 1962; Jones, 1962; Harvey et al., 1963). It is the over-all purpose of this study to elucidate the basis for this disagreement.

Suggestions for lines of approach to the problem of uncovering the sources of disagreement concerning the intracellular distribution of CH are supplied by consideration, first, of the cell structures involved in the controversy, and, second, of the various methods of deriving cellular material for observation.

The cell structures predominantly involved in the disagreement over intracellular localization of CH are the nucleus and mitochondria. It has recently been shown, relative to the nucleus, that desoxyribonucleic acid (DNA), a major component of that cell structure, is an effective quencher of CH fluorescence (Boyland and Green, 1962b). Under the in vitro conditions of Boyland and Green's study, pH and ionic conditions of the DNA solutions had marked effects on the ability of DNA to quench, minor increments in ionic strength at physiologic pHs causing a reduction in quenching. This variable effect of DNA on the fluorescence of CH disallows a prediction of the effect of CH within the living cell and requires a direct study of the effects of intranuclear DNA on the observed patterns of fluorescent hydrocarbons.

Mitochondria are structures containing high concentrations of lipid (Novakoff, 1960), a class of intracellular substances reputed to play a major role in determining accumulation of hydrocarbons by the various cell structures (Berg, 1951/1952). This same author has

proposed that CH, with a high degree of sensitivity and specificity, exclusively demonstrates specific sites of intracellular lipid. Berg's hypothesis, in addition to being in disagreement with the results of observations on mitochondria in living cells under physiologic conditions, is also possibly in disagreement with the finding of a number of biochemists that CHs are strongly complexed with cellular proteins (Miller, 1951; Weist and Heidelberger, 1953a, b and c; Moodie et al., 1954; Woodhouse, 1954, 1955; Bhargava et al., 1955; Bhargava and Heidelberger, 1956; Heidelberger and Moldenhauer, 1956; Darchun and Hadler, 1956; Carruthers et al., 1957; Hadler et al., 1957; Olivero and Heidelberger, 1958; Davenport et al., 1961; Somerville and Heidelberger, 1961; Abel and Heidelberger, 1962). In light of Berg's study (1951/1952), an evaluation of the role of lipid on the intracellular distribution of CH is indicated.

Differences in methods employed by the many workers in the past and present preparing cellular material for determination of the intracellular sites of distribution of CH have not been adequately evaluated as to their influence on CH distribution.

1) The method of subcellular fractionation of cells exposed to CH followed by CH determination carried out on the separated cell fractions has been commonly used (Weist and Heidelberger, 1953a, b and c; Calcutt and Payne, 1954, 1954; Woodhouse, 1954; Fiala, 1959). Norden (1957a), in a limited study of the patterns of CH in liver nuclear fractions, noted variation in the pattern of nuclear uptake of CH with the type of medium used in isolation. No further investigation into factors of this variation has been undertaken.

2) The method of fixation prior to investigation of the intracellular distribution of CH has been frequently utilized (Graffi, 1941/1942; Günther, 1941/1942; Hamperl et al., 1942; Simpson and Cramer, 1943a and b, 1945; Ahlström and Berg, 1947, 1949; Ahlström, 1949; Setälä and Ekwall, 1950a and b; Ekwall et al., 1951; Ermala et al., 1951; Cambel, 1951; Setälä, 1952; Berg, 1951/1952; Nordén, 1952, 1953a, 1957a). The results of these studies are generally in limited agreement with the studies of the intracellular distribution of CH in living cells, with regard to the nucleus, but not, as previously pointed out, with regard to the mitochondrial complement. Previous studies of fixation effects on intracellular patterns of CH have been confined to a comparison of frozen-sectioned, unfixed cells with frozen-sectioned, fixed cells (Simpson and Cramer, 1945; Ermala et al., 1952; Setälä, 1951; Berg, 1951/1952; Nordén, 1952, 1953a). Only one limited study involving direct comparison of living with whole fixed cells has been reported (Günther, 1941/1942).

3) In study of living whole cells under in vitro conditions with u-v-fluorescence microscopy, there has been difficulty in obtaining whole cells in normal physiologic condition (Shires, 1962; Giovanella and Heidelberger, 1965). Recourse to study of the intracellular distribution of CH in tissue culture cells has been undertaken (Richter and Saini, 1960; Harvey et al., 1963). Extensions of these studies to include an investigation of the patterns of intracellular CH under a variety of experimental conditions and throughout the cytologic modifications of the cell during the growth cycle have not been

undertaken.

To elucidate the basis for the apparent discrepancies existing in the literature relative to the intracellular sites of accumulation of CH, this study has been directed toward the following specific, mutually dependent objectives: 1) the extension of present knowledge of the intracellular distribution of CH and non-CH in living cells under various experimental conditions designed to effect alterations in the physiologic state and in various phases of the mitotic cycle, 2) the evaluation of effects of fixation and routine methods of microtechnique on the pattern of intracellular CH as compared with those patterns observed in the living cells, 3) the evaluation of the pattern of CH uptake of liver nuclei observed in various routine isolation media and conventional buffer solutions, 4) the evaluation of the role of nucleic acid in influencing the intracellular distribution of CH in living cells, 5) the evaluation of the role of general protoplasmic protein on the intracellular pattern of BP, and 6) the evaluation of the role of lipid on the intracellular distribution of CH.

CHAPTER II

THE GENERAL PLAN FOR THE ELUCIDATION OF PROBLEMS

Biological Test Material

This study has centered about two different cell types: the Strain L-929 fibroblast and the hepatic parenchymal cell of the rat (King-Holtzman).

The cells of the liver were used in two separate studies: those involving ultracentrifugation of living cells and the investigation of subcellular fractions. The ultracentrifugation study required a tissue which could be experimentally treated and then dissociated into whole cell suspensions. Of several tissues tried, including epididymis, choroid plexus, kidney and liver, only the liver could be successfully handled. The use of liver as a source of subcellular fractions was dictated by the large amount of general research carried out on liver cell fractions and the fact that most fractionation techniques have been developed on liver cells (Dounce, 1950, 1955; Siebert and Smellie, 1957; Roodyn, 1959, 1963).

The Strain L-929 fibroblasts used in this study was derived from a clone of L-strain fibroblasts in their 95th passage in heterologous medium (Sanford et al., 1948). Strain L-929 fibroblasts

(L-cells) were chosen for this study because (1) their rapid growth rates in vitro provided a plenitude of whole intact living cells for study, 2) the fibroblasts growing on glass substrates were flattened, providing excellent optical conditions for cytologic observations, 3) the effects of direct exposure of actively dividing cell populations to carcinogens and non-carcinogens and other agents could be directly observed, and 4) the monolayers on glass substrates could be conveniently handled in making live cell preparations and histochemical analysis of those cells.

Strain L-929 fibroblasts represent highly modified cell types. Since establishment of the L-929 clone, the cells have developed the ability to give rise to sarcomas after innoculation into the C3H mouse strain of origin (Sanford et al., 1950, 1954, 1956) and to induce immune reactions in the same mouse strain (Algire et al., 1950; Sanford et al., 1950, 1956). Further alterations in sarcogenicity, histocompatibility and physiology were attendant on conversion to serum-supplemented synthetic media (Sanford et al., 1959). Modification of cell types has been detected in most long-term cultured cell strains and appears to represent the typical reaction of living cells to prolonged habitation in in vitro conditions (Gey and Gey, 1947; Goldblatt and Cameron, 1953; Leighton et al., 1956; Coriell, et al., 1957; Sanford et al., 1959; Earle, 1961; Staroverova, 1961; Staroverova and Vasiliev, 1962; McAllister, 1962).

The In Vitro Systems

The L-929 fibroblasts and liver cells were utilized in the preparation of three types of *in vitro* systems: 1) monolayer cultures of L-929 cells, 2) living intact liver cell suspensions, and 3) subcellular fractions of liver cells.

L-929 Culture Methods and Preparation of Experimental *In Vitro* Systems

Routine stocks of strain L-929 fibroblasts (Microbiological Assoc., Inc.) were carried in MEM or Medium 199 with 10% horse serum and 5,000 units per ml. of a penicillin-streptomycin mixture (all from Microbiol. Assoc., Inc.) for a year and a half from their date of purchase in 1961. At that time additional cells were received from the same firm, and the stocks were switched to BME with 10% horse serum supplement and 5,000 units of the penicillin-streptomycin mixture (all from Flow Labs., Inc.). The stocks were customarily seeded in 10 cc. aliquots of medium containing a total cell population of two to three million cells (viable cell count) and maintained in plastic top, glass milk bottles (Kimax) at a constant temperature of 37°C by a thermomonitor (Sargeant). After development of complete monolayers (generally at the end of three to four days), cells were trypsinized by the procedure of Dulbecco and Vogt (1954) using Difco's "Bacto-Trypsin" (1:250) and resuspended in fresh medium for reseeding for the maintenance of stock cultures and for experimental studies.

Experimental studies were carried out on coverslip cultures, grown in disposable, plastic petri dishes (30 mm. - Falcon Plastics

Corp.) or in Cooper dishes (Falcon Plastic Corp.). They were set up by adding 1-1 1/2 cc. or 3-3 1/2 cc., respectively, of an L-cell suspension (containing approximately 200,000 viable cells per cc.) to the culture vessels containing each, a 22 mm. square glass coverslip. The disposable dishes were sealed with silicone grease (Dow Corning) and incubated up to four days at 37°C.

The *In Vitro* Systems of Whole Liver Cells

King-Holtzman rats were sacrificed by subluxation of the cervical vertebrae, the abdominal cavity was opened and small fragments of liver were excised and added to complete growth medium (BME or MEM) in a physiologically clean petri dish. Fragments were minced with scissor-type strokes of knives fitted with #11 Bard Parker blades until a turbid suspension of whole cells was obtained.

The *In Vitro* System of Liver Cell Fractions

Subcellular fractions of King-Holtzman rat livers were prepared by a variety of procedures (Dounce, 1943b and c; Schneider *et al.*, 1948; Dounce, 1950; Schneider and Peterman, 1950; Calcutt and Payne, 1954; Hogeboom *et al.*, 1952; Chauveau *et al.*, 1956). The general plan of preparation was to quickly sacrifice the animal; open the abdomen by a midline incision, carefully avoiding contamination of the viscera with hair; excision of small fragments of tissue and their suspension in appropriate ice-cold media. Both a glass, ball-type homogenizer (Dounce, 1955) and a VirTis homogenizer were used to break up the tissues and cells. The homogenate was differentially centrifuged and subcellular fractions obtained, but the nuclear fraction, being of

main interest, was the only fraction kept. Stock nuclear suspensions were prepared for experimental purposes by resuspending centrifugally sedimented nuclear pellets in an equal volume of appropriate medium. For microscopic observation, the three types of *in vitro* systems, as monolayer cultures, liver cell suspensions, and liver subcellular fractions were set-up by established methods for a) wet microscopic slide preparations (Richter and Saini, 1960) and for b) dry or permanent slide preparations (Lillie, 1948; Gridley, 1960).

The General Analytic Observational Method

The general analytic-objective method used to determine the incorporation of hydrocarbons in the several *in vitro* protoplasmic systems already noted involved 1) the exposure of the biologic material to fluorescent hydrocarbons and 2) its examination via an integrated phase-contrast and u-v-fluorescence microscopic technique to obtain direct visual and photographic data on the distribution pattern of fluorescent hydrocarbons.

Hydrocarbons Studied and Methods of Introducing Them To The *In Vitro* Protoplasmic Systems

The carcinogenic hydrocarbon used throughout the study was 3:4 benz(a)pyrene (BP) (Hoffman LaRoche); the non-carcinogenic hydrocarbon used was pyrene (Py) (Eastman). Both hydrocarbons are polycyclic structures, exhibiting blue fluorescence as crystals and in molecular solutions, and yellow when in colloidal suspension (Weigert and Mottram 1940; Weigert, 1942; Rodd, 1956). The solubilities of these

hydrocarbons in aqueous solutions are extremely low and difficult to measure, perhaps running about 1 ugm. per cc. (Earle and Voeglin, 1938; Hollaender et al., 1939; Peacock, 1940; Mueller and Rusch, 1943; Haddow, 1947; Rodd, 1956).

The general method of introducing hydrocarbon to the protoplasmic systems in every situation involved direct solubilization of crystalline material in aqueous media. Despite the low solubility, water solvents have been demonstrated to be adequate in conveying hydrocarbon from crystals to cells (Richter, 1951, 1952a and b, 1955; Richter and Saini, 1960; Shires, 1962; Jones, 1962; Harvey et al., 1963). Both CH and non-CH were introduced to the in vitro protoplasmic systems on crystalline beds. The amount introduced was that which crystallized out of a saturated ethanol solution of hydrocarbon contained within a wire-loop 2 mm. in diameter. The exposure of the in vitro systems to the hydrocarbon was experimentally varied as follows.

Relative to living cell cultures, two methods of exposing strain L-929 fibroblasts to CH and non-CH were used. In the first method, L-cells were grown directly on hydrocarbon crystals by seeding cell suspensions on top of a prepared coverslip which had been placed, under sterile conditions, in a disposable culture dish. These coverslip cultures were then sealed and incubated as previously indicated. For microscopic study, the coverslips containing the exposed monolayered cells were mounted on clean glass slides in complete physiologic media and sealed in beeswax.

In the second method of exposing living L-cells to CH and non-CH, crystalline beds of hydrocarbon were prepared on physiologically clean glass slides. Coverslip cultures containing living fibroblasts were inverted onto the crystalline bed in complete growth medium. The preparation was then sealed by ringing the edges of the coverslip with beeswax to avoid dessication. Uptake of the CH was rapid and allowed immediate observation. In the case of pyrene, uptake was slow and incubation of 15-20 minutes at 37°C was required.

Relative to living liver cell suspensions: exposure of suspensions of whole liver cells to CH was effected by pipetting a drop of the suspension onto glass slides on which crystalline beds of BP were previously formed. The preparation was covered with a clean coverslip and immediately sealed with beeswax. Direct observation could be undertaken as soon as the slides were prepared because of the rapid uptake of the CH by the hepatic cells.

Relative to subcellular fractions: suspensions of liver nuclei were applied to crystalline beds of BP by pipette. A coverslip was dropped on the preparation carefully to avoid the entrapment of air bubbles, and the preparation was sealed by ringing with beeswax. The CH was rapidly incorporated by the nuclear fraction, and thus, ready for immediate observation.

All preparations of biologic materials exposed to CH or non-CH were controlled by identical preparations containing no hydrocarbons. All other steps in the preparation of controls were similar to those used for the experimental analysis of hydrocarbon distribution.

The Observational Method

Study of the in vitro protoplasmic systems was carried out by an integrated phase-contrast and u-v-fluorescence microscopic procedure (Richter, 1951, 1952a and b, 1955: Richter and Saini, 1960).

Phase-contrast equipment consisted of an American Optical dark medium phase-contrast microscope, and the American Optical incandescent lamp #735 with a filter of 5% sodium nitrite.

U-V-fluorescence microscopy was done with an A. O. Spencer Research microscope, equipped with quartz substage condenser, with ordinary and reflecting objectives and with quartz oculars. Light, provided by an Osram HBO-200 mercury arc lamp, was transmitted through a Corning Filter #5840 which gave maximal transmission of light at 300-425 millimicra. Two types of u-v absorption filters were also employed: Corning #3387 and a 5% sodium nitrite aqueous solution, and two types of infrared filters: Corning #3966 and a 5% copper sulfate solution.

This integrated microscopic procedure was employed to directly observe the results of experimentation on living L-cells, whole liver cell preparations and liver nuclear fractions set up on slide preparations. In practice, the preparations were initially studied with phase-contrast microscopy, specific cells or nuclei located and the coordinate on the microscope's mechanical stage noted. On transfer to the u-v-fluorescence microscope, the selected cell or nucleus was found by adjustment of the mechanical stage to the appropriate coordinates.

Protection against light damage to biological material was

afforded by use of u-v and infrared filters during phase-contrast microscopy and by limiting the time for observation (and photography) of any one preparation to no more than a total of 20 minutes.

The utility of this integrated phase-contrast and u-v-fluorescence microscopic method for studying the patterns of hydrocarbon uptake lies 1) in its applicability to living, experimentally treated cells or cell fractions, and 2) by means of the integrated system, identification of specific protoplasmic sites relative to CH distribution can be readily and accurately accomplished.

Major Experimental Lines of Investigation

Consistent with the specific objectives stated earlier, this study has progressed along the following major lines:

Experiments bearing on the Patterns of CH and non-CH Accumulations in Living In Vitro Cell Systems

Study of the distribution of CH within living Strain L-929 fibroblasts and intact hepatic parenchymal cells and within isolated liver nuclei was directed toward observation of the relative hydrocarbon accumulation in specific cytoplasmic components (e.g. mitochondria and elements of the Golgi complex) and nuclear structures (e.g. nucleolus, nuclear membrane and chromatin). Correlative histochemical and conventional staining procedures were done to determine the identity and character of the various cell structures. These studies on the apparent intracellular distribution of CH were accompanied by parallel studies on the pattern of incorporation of a non-CH. The establishment

of data concerning patterns of incorporation of CH and non-CH within living L-cells, whole hepatic parenchymal cells and isolated liver nuclei is essential as a reference for the other facts of the over-all study and also represents an extension of the total knowledge of the distribution of these hydrocarbons within living cells.

Experiments Bearing on the Influence of Routine Processing Agents of Microtechnique on the Distributions of CH and non-CH in Strain L-929 Fibroblasts

A study on the influence of some routine processing agents of conventional microtechnique on the intracellular distribution of fluorescent CH was carried out on L-929 fibroblasts. The effects of seven single-agent and five multiple-agent fixatives and of freezing were considered. The additional influence of such post-fixation procedures as dehydration and clearing or the removal of mercury from cells fixed with $HgCl_2$-containing fixatives was also studied. The intracellular pattern of Py in cells fixed with the single-agent fixatives was carried out for comparison with the pattern of BP within cells fixed with the same agents.

Several types of experiments were performed in studying fixation effects on the intracellular patterns of the hydrocarbons. 1) The effects of length of fixation and pH of fixative were explored. 2) The effects of fixing cells already containing BP (L-cells grown on BP beds) were compared with the effects of fixing cells prior to exposing them to crystalline beds of BP. 3) The intracellular pattern of BP in cells fixed with a complex fixative was contrasted with the

patterns within cells fixed in its various components, either singly or in combination.

Observation was concerned with acquiring data about the status of intracellular BP or Py in various cell components and the recognition of characteristic patterns of intracellular CH or non-CH distribution in cells treated with particular fixatives. These patterns of hydrocarbon uptake could then be compared with those derived from integrated phase-contrast and u-v-fluorescence observation of living cells.

Experiments Bearing on the Distribution of CH in Isolated Liver Nuclei

The object of this study was essentially to determine the influence of various nuclear media on the uptake of BP by these structures when in isolation. The media under consideration fell into three categories: A) those media involved in some six different conventional isolation procedures, B) four different buffers at a variety of pHs and C) some conventional nuclear isolation media without the addition of $HgCl_2$. Observations on nuclei in any of these three types of media were concerned with the nuclear rim, nucleoli and the chromatic and achromatic interphase structures, in regard to their relative apparent affinities, per se, for BP.

As control over the condition of nuclei suspended in the various media, several standard tests were made. 1) A histochemical test for the presence of DNA was carried out to determine its presence or absence in nuclei treated with the various media. 2) A gel formation

test was done to provide a crude estimate of the condition of the nucleoprotein within the nuclei (Roodyn, 1963). 3) In the case of nuclei suspended in the different buffer solutions, the pH of the nuclear suspension was measured. This latter procedure was carried out when it was found the pH of the nuclear suspension was quite different from that of the buffer before nuclei were suspended in it.

Data concerning the distribution of CH in isolated nuclei under this variety of conditions were compared with the distribution pattern of CH relative to the nuclei in whole, living hepatic cells.

Experiments Bearing on the Influence of Nucleic Acids on the Intracellular Distribution of Carcinogenic Hydrocarbons

Study of the influence of nucleic acids on the pattern of BP uptake by living cell components was approached by three general procedures: 1) selective removal of nucleic acids from whole L-929 fibroblasts, 2) selective removal of nucleic acids from isolated liver nuclei and 3) the intracellular displacement and stratification of nucleic acid rich structures of living hepatic cells by ultra-centrifugation of hepatic tissue. U-V-fluorescence microscopic observation was made after experimentally-treated whole cells or cell fractions had been exposed to crystalline beds of BP on glass slides.

The nucleus was a special object of study. Its content of DNA is of special concern as a possible quencher of intracellular CH fluorescence (Boyland and Green, 1962b). It is the largest, and only, intracellular structure which has consistently been observed to demonstrate

no CH uptake in living cells (Graffi and Maas, 1940; Günther, 1941/1942; Waddington and Goodhart, 1949; Richter, 1951, 1952a and b, 1955; Richter and Saini, 1960; Shires, 1962; Jones, 1962; Harvey et al., 1963). The nucleus is thus an especially useful test object for evaluation of the quenching effect of DNA on CH fluorescence.

Studies on the Influence of Protein and Lipid on the Intracellular Distribution of Carcinogenic Hydrocarbon

Total extraction of cellular lipid was carried out on L-929 fibroblasts to ascertain if 1) after lipid removal, the extracted cells would show uptake of BP either from crystalline beds or from solutions in which BP was made more soluble in water by caffeine (Berg's solution 1951/1952), and to determine if 2) fluorescence hydrocarbon in BP-containing cells could be taken out with the lipid solvents.

The first study has bearing on the influence of lipid on the intracellular distribution of CH and, also, on the contention by Berg (1951/1952) that lipid is the major factor in accumulating BP at particular cell sites. This study proposes to exhaustively extract lipid from L-cells, not grown on BP, with solvents to remove most of the intracellular lipid. The extracted cells are then to be exposed to crystalline beds of BP and observed for evidence of intracellular uptake and distribution of BP by the various cellular structures.

The second study involved exhaustive lipid extraction of L-cells grown on BP. The existence of fluorescent material resistant to extraction with lipid solvents was expected to indicate CH bound to protein or nucleoprotein, forming a complex known to be fluorescent

(Miller, 1951; Moodie et al., 1954; Woodhouse, 1954, 1955; Carruthers et al., 1957). Treated cells were then mounted on clean glass slides and observed for the presence-absence of fluorescence.

These combined procedures were designed to assess the influence of lipid in determining the observed patterns of apparent intracellular CH, in extension of previous studies which have reported that it did. The action of proteins, which bind CH, in effecting intracellular BP distribution was investigated.

CHAPTER III

THE MORPHOLOGIC, CYTOCHEMICAL AND BIOLOGIC CHARACTER OF THE STRAIN L-929 FIBROBLAST AND HEPATIC CELL

Introduction

Prior to study of the L-929 fibroblasts or hepatic parenchymal cells as test objects, their phase-contrast cytology was established. Structural data obtained on the living cells was correlated with results on fixed cells treated by routine histochemical methods.

Materials and Methods

The methods of preparing live cell preparations for microscopic observation have been described above.

Histochemical and routine staining procedures were carried out on liver and cultures fixed in Lillie's buffered 4% formaldehyde (Lillie, 1948).

L-cells in coverslip cultures were fixed by immersing the cell-containing coverslips in fixative directly in a Columbia staining dish. All chemical and staining procedures were carried out on the fixed cell-containing coverslips by immersion in Columbia staining dishes containing the appropriate fluids.

Liver tissues, fixed for 6 to 24 hours were dehydrated in graded alcohols, cleared in toluene and embedded in paraffin blocks by routine procedure. Sections were cut at approximately 10 micra and histochemical and routine staining procedures carried out on the sectioned material.

Observations

Morphologic and Histochemical Consideration of the L-929 Fibroblast

Since Dalton and Earle's publication (1944), no major cytological study of the L-929 fibroblast, or its L-strain precursor, has appeared in the literature. The transmutations of these cells have only been recorded in low-power photomicrographs (Earle et al., 1950) and these descriptions continue to be applicable to lightly seeded L-929 fibroblasts in MEM, BME or NCTC 199 (Figs. 7, 8, 10, 12, 14, 28, 31, 35). On glass substrate, the form of L-cells was predominantly stellate with fewer spindle-shaped cells (Figs. 4, 6), and giant cells of the mononuclear or polynuclear varieties (Figs. 48, 49, 93). Intercell-contact generally occurred at the tips of cell processes, becoming increasingly broad as the population increases (Figs. 10, 12, 31, 32, 33, 34, 35). During and through the logarithmic growth phase, mitotic cells appear to lose their contacts with other cells (Figs. 17, 19, 21, 22, 24, 77). The apparent syncytial relationship of long-term cultured fibroblasts (Fig. 10) has been denied (Gey, 1954/1955; Davis and James, 1962).

The fibroblast cytoplasm may be divided into three regions (Frédéric, 1958): a central region, containing the nucleus and Golgi zone; a peripheral cytoplasmic region, extremely thin and involved in all cell processes; and an intermediate region, containing most of the mitochondria and of variable thickness in between the thick central and thin peripheral regions.

L-cells, like other fibroblasts in vitro, had three basic forms of cell processes. Large processes, finely pointed or blunt, and containing a core of intermediate zone cytoplasm, were a feature of most non-mitotic cells (Figs. 1, 4, 6, 7, 8, 10). Velamentous processes with broad, often ruffled, fronts (Figs. 1, 4) were similar in appearance to those reported associated with pinocytosis (Lewis and Lewis, 1924; Lewis, 1937; Gey et al., 1953/1954; Gey, 1954/1955), although non-pinocytotic cells may also display them (Lewis, 1937; Porter et al., 1945). Fine cytoplasmic processes, appearing like long spikes leaving the peripheral cytoplasmic surface at right angles and only a few tenths of a micron wide on interphase (Fig. 7) or mitotic cells (Fig. 21), have been frequently reported on avian and mammalian fibroblasts in vitro at the light level (Lewis, 1917; Gey et al., 1953/1954; Gey, 1954/1955; Frédéric, 1956) and ultrastructural levels (Porter et al., 1945; Borysko and Sapronauskes, 1954; Selby et al., 1956; Porter and Pappas, 1959; Causey and Heyner, 1963). The resemblance of these fine cytoplasmic processes to the microvilli of epithlial cells has been noted even in the case of the L-cell (Bensch et al., 1964).

L-929 fibroblast mitochondria tend to be granular or rod-shaped

(Figs. 1,6) rather than the long filamentous shape described in primary explant fibroblasts (Lewis, 1917; Lewis, 1919; Lewis and Lewis, 1924; Bloom, 1936; Porter et al., 1945; Ludford et al., 1948; Fish, 1950; Davies, 1952; Porter and Kallman, 1952; Motohiko and Biesele, 1956; Frederic, 1956, 1958; Richter and Saini, 1960; Causey and Heyner, 1963) and fibroblast strains in long-term cultures (Carrel and Ebeling, 1926; Dalton and Earle, 1944; Gey et al., 1949; Ludford and Smiles, 1950; Gey et al., 1953/1954; Gey 1954/1955). L-cell mitochondria were less than 0.6 micra in diameter and rarely more than 1 micron in length. Staining of L-cells with Regaud's mitochondrial stain (Gatenby and Beams, 1950) revealed the occasional occurrence of mitochondria in clumps 1.2 micra and more in width. Most mitochondria were found in the intermediate cytoplasmic region, but not infrequently penetrating the Golgi zone (Figs. 7,8) or lying within the larger cytoplasmic processes (Fig. 1) and velamentous cytoplasmic processes (Fig. 4). Rod-shaped mitochondria were usually oriented parallel to the cell axis (Figs. 1, 6).

Discrete juxtanuclear Golgi zones, occurring at the nuclear pole, have been described in a wide variety of in vitro fibroblasts after various classical Golgi techniques (Zweibaum and Elkner, 1926, 1930; Ludford, 1927; MacDougald, 1937; Dalton and Earle, 1944) in phase-contrast microscopic studies of living fibroblasts (Ludford and Smiles, 1950; Frédéric, 1956; Richter and Saini, 1960) and with the electron microscope (Porter et al., 1945; Selby et al., 1956; Schwarz et al., 1962; Causey and Heyner, 1963; Bensch et al., 1964; Goldberg

and Green, 1964). In detail, the zone was composed of an aggregation of refringent granules, less than 0.6 micra in diameter, and occasional granular or rod-shaped mitochondria (Figs. 7, 8). By random count (500 cells in 10 cultures) only 33% of the L-cells had discrete juxtanuclear Golgi zones. In the remainder of cells, the Golgi element was represented by dispersed Golgi granules, small angular structures (Porter et al., 1945; Richter, 1956; Hayward, 1961) ranging in size from 0.2 to 1.2 micra (Figs. 1, 4, 6, 12, 21) and stained lightly with the Kolatchew-Nassonov Golgi stain (Gatenby and Beams, 1950) and with Baker's acid hematein test (1946) for phospholipid. Dalton and Earle (1944) reported the Golgi granules originated from fragmentation of Golgi zone material during cell degeneration. Hayward (1961) reported that the dispersed Golgi form is the characteristic one in L-cells.

Threads of fibrous material of variable lengths were encountered with phase-contrast microscopy apparently at or near the surface of L-929 fibroblasts (Figs. 4, 7, 8), as previously reported from light and electron microscopic studies (Kuchler et al., 1960; Richter, 1965). The threads run a course which sometimes parallels the long axis of protoplasmic processes and which may be seen to intersect with the fine cytoplasmic processes (Figs. 7 and 8). The fibrous material failed to stain with Mallory's phosphotungstic acid-hematoxylin (Mallory, 1904), Gomori's reticulin stain (Gridley, 1960), or Van Gieson's collagen and Verhoeff's elastin stains (Gridley, 1960). Regaud's iron Hematoxylin (Gatenby and Beams, 1950) blackened the fibrous material, and the Mercury Bromphenol-Blue stain for protein

amino groups (Mazia et al., 1953) stained the material an intense blue. Thus, these cellular fibers, while morphologically resembling Mallory's "fibroglia" (1904), Maximow's "tonofibrils" (cited in Porter and Pappas, 1959), Lewis's "collagen fibers" (1917) and Merchant and Kahn's "collagen" (1958), possess staining characteristics similar only to the "tonofibrils".

Porter and Pappas (1959) have likened "fibroglia" and "tonofibrils" to the ridges of amorphous material or fibers found by electron microscopists on the surface of fibroblasts engaged in the in vitro production of collagen (Porter and Pappas, 1959; Kuwabara, 1959; Kuroki, 1960; Yardley et al., 1960; Schwarz et al., 1962; Goldberg and Green, 1964). However, judging by the very low hydroxy-proline content of L-cell cultures, it has been reported that Strain L-929 cells probably do not produce collagen (Goldberg et al., 1963), and thus, the nature of the fibrous material, except for its protein content, remains unestablished.

Studies of fibroblast lipids have been few in number, and lipid characterization of L-cells (Geyer et al., 1962) has been made as a comparison with other tissue culture strains, many epithelial in origin. Histochemically, L-cell lipid was poorly resolved, showing little or no coloration with Sudan Black B (Kahn et al., 1962), a saturated mixture of Sudan III and IV (Pearse, 1960) and after the plasmal reaction (Hayes, 1949). Only a variable, light blue staining was obtained with Nile Blue (Cain, 1947), and no particular cytoplasmic localization was noted. Baker's acid Hematein (Baker, 1946) gave distinct coloring

to pyridine-extractable material in the central cytoplasmic region, including the Golgi zone, and to peripheral Golgi granules.

The occurrence of lipid droplets of high refractility in fibroblasts under special conditions of culture (Biesele and Golghaber, 1955; Chèvrement et al., 1956; Richter, 1956; Wilson, 1961; Rose et al., 1961; Krygier, 1962) and as routine cytoplasmic inclusions (Biesele and Goldhaber, 1955; Davies, 1952) has been reported, but nothing quite comparable to these lipid droplets was observed in routine cultures of L-cells.

The nuclei of L-cells were usually spherical to oval in shape. Three general heterochromatic regions were discerned with the Feulgen reaction: nucleolar-associated chromatin, forming an almost complete nucleolar covering; a nuclear membrane-associated chromatin, underlying the nuclear membrane with few breaks in continuity; and a finely granular chromatin, situated between nucleolus and nuclear rim (Figs. 1, 7, 8, 10). These chromatin granules were well under 0.6 micra in size and were arranged in strands, isolated, or connected with the nuclear membrane or nucleolus, or as apparently isolated granules. All the heterochromatic regions were also stained with Gallocyanin-Chrome Alum (Einarson, 1951), after DNAase or RNAase hydrolysis, and with the Bromphenol-Blue stain for peotein. Gallocyanin-Chrom Alum staining, after digestion with DNAase, revealed that the presumtive RNA occupied the same intranuclear sites as DNA (Caspersson, 1947; Ludford et al., 1948a and b; Kaufman et al., 1951; Butler et al., 1956; Barka and Dal Dallner, 1959).

Nucleoli were either very large irregular structures, numbering one to two per nucleus (Figs. 1, 6, 7, 8, 10, 32, 35) or smaller, more compact entities numbering two to five or more (Figs. 6, 8, 12) but variable, in that some cultures might have 100% of their nucleoli vacuolated while another would have none. No particular reason for this variation was discovered. The size of the vacuoles was also variable, generally ranging around 0.6 micra and rarely becoming as large as 1.8 micra. Filamentous elements in nucleoli are reported in the nucleoli of living fibroblasts and other cells (Hugh, 1952; Zajdela and Morin, 1952; Denues and Mottram, 1955; Guttes and Guttes, 1961) were never distinctly observed in L-cells. Occasional dark knobs on the nucleolar surface (Figs. 1, 8) probably correspond to segments of nucleolus-associated chromatin (Caspersson, 1947; Ludford et al., 1948a and b; Ludford, 1954.)

Most cultures of L-929 fibroblast contain some cells with pleomorphic nuclei (Fig. 84) nuclear fragments (Fig. 1) and are in a bi-or multinucleate condition (Fig. 93), as reported from many strains of cultured cells (Lewis, 1947 and 1951; Bucher and Gattiker, 1953; Ludford, 1954; Hsu and Moorland, 1955/1956; Nagata, 1962; Longwell and Yerganian, 1965). An apparently as yet unreported feature of L-cell nuclei was the appearance of tongues of cytoplasm, penetrating, sometimes quite deeply, into the nucleus (Figs. 48 and 49). When these structures lay in a horizontal plane, as was common in large mononuclear cells, their cytoplasmic origin was clear; however, much more commonly, they apparently lay in a vertical plane, appearing as

non-nuclear elements deep inside the nucleus (Fig. 10). No discrete nuclear membrane appeared to circumscribe them, nor was it possible to determine if they were all in continuity with the main cytoplasm. Structures of perhaps some similarity have been reported from other proliferative cell populations in the liver and in tumors (Kleinfeld et al., 1956; Binggeli, 1959; Leduc and Wilson, 1959). Development of the centrosomal region, routinely observed in many other cultures of fibroblast types (Fell and Hughs, 1949; Lettré et al., 1951; Lewis, 1951; Boss, 1954a), was not detected in the polar cytoplasm.

After dissolution of the nucleolus and nuclear membrane, the metaphase plate was formed of chunky masses of chromosomal material in which individual chromatid pairs were not discernible (Fig. 17). The plate lay at right angles to the glass substrate surface, in common with other metaphase plates of mouse but not avian origin (Fell and Hughes, 1949). Perichromosomal cytoplasm, relatively clear of visible inclusions, surrounded and penetrated the plate (Fig. 17). No spindle or astral structures were detected during metaphase or early anaphase, but as the daughter chromosomes reached a later stage axially oriented fibers became visible in the interzonal spindle (Fig. 27) and remained visible into the telophase (Fig. 28). Daughter chromosomes throughout anaphase retained their clustered array with little perichromosomal cytoplasm separating them (Figs. 19, 21, 22, 24, 25, 27).

In the cytoplasm throughout the mitotic cycle, presumptive Golgi granules and mitochondria were observed (Figs. 14, 17, 19, 21,

23, 25, 27, 28, 30). The fine cytoplasmic processes, previously noted in inter-mitotic cells, continued to be found emanating even from spherical mid-mitotic cells (Figs. 17, 19, 21, 22). Characteristic surface blebbing of mitotic cells (Boss, 1955; Fell and Hughs, 1949; Lettré et al., 1951; Lewis, 1951; Davies, 1952) occurred from metaphase through early telophase (Figs. 17, 22, 24, 27, 28, 30). In late anaphase or early telophase the equatorial grooves of cytokinesis developed (Figs. 22, 24, 25, 27), but equatorial plates, as distinct cytoplasmic structures, were unnoticed until later telophase (Figs. 28, 30).

In the telophase reconstruction of the nucleus, daughter chromosomes swelled and showed differentiation into apparent heterochromatic (phase-dark) and euchromatic (phase-light) regions (Figs. 28, 30). Lateral association of the swelling karyomeres and loss of interkaryomeric cytoplasm, as described by Lewis (1947 and 1951), Fell and Hughs (1949) and Ludford (1954), formed an aggregate, on the perimeter of which the nuclear membrane reformed (Figs. 28, 30).

Encounter with evidently bizarre mitosis, such as multipolar divisions, was occasionally observed in every culture, in agreement with observations on other fibroblast-like strains grown in vitro and even fibroblasts from freshly explanted tissues (Lewis, 1947, 1951; Fell and Hughs, 1949; Bucher and Gattiker, 1953; Gey et al., 1953/1954; Gey 1954/1955; Hsu and Moorhead, 1955/1956; Nagata, 1960; Krygier, 1962). Recent karyological study of L-929 fibroblasts established them as variably aneuploid with modal values of 60 to 70

chromosomes (compared with 40 chromosomes for the C3H mouse of origin - Hsu, 1962), but similar chromosomal instability has also been demonstrated in chick embryo fibroblasts during the first and second passages through synthetic media (Krygier, 1962).

Morphologic and Histochemical Considerations of Isolated Whole Hepatic Parenchymal Cells

Hepatic parenchymal cells, observed with phase-contrast microscopy in live cell, in vitro slide preparations, contained one or two approximately spherical nuclei of somewhat variable sizes (Mann, 1928; Beams and King, 1942; Zollinger, 1948; Lagerstedt, 1949; Harrison, 1953; Fawcett, 1955; Kasten, 1958). Feulgen-stained chromatin variably covered the one to several nucleoli of the hepatic cell and occurred as irregular strands, often interconnecting the nucleolar-associated chromatin and chromatin lining the inner surface of the nuclear membrane (Figs. 231, 233, 235, 237) (Brenner, 1953; Lagerstedt, 1949; Baradwaj and Love, 1959; Leduc and Wilson, 1959; Bucciolini and Marsilii, 1960; Colosi and Marsilii, 1960). The mitochondrial complement which was uniformly distributed throughout the cytoplasm, consisted of filamentous (Fig. 233), granular and vesicular forms (Fig. 237) (Nöel, 1923; Steffens, 1941; Deane, 1944; Deane and Green, 1946; Lagerstedt, 1949; Fawcett, 1955, Bucciolini and Marsilii, 1960; Colosi and Marsilii, 1960). The Golgi complex was not certainly identified, but an irregular formation of threads and vesicles was found situated in the vicinity of the cell nucleus (Figs. 233, 235, 237) or near the cell surface (Fig. 231) (Bowen, 1926; Nassonov, 1926; Cramer and

Ludford, 1926; Dalton, 1934; Deane, 1946; Fawcett, 1955; Karrer, 1960). One to several frank lipid droplets occurred randomly placed in the cytoplasm (Figs. 231 and 233) (Zollinger, 1948; Gatenby and Moussa, 1951; Bucciolini and Marsilii, 1960; Colosi and Marsilii, 1960; Mateyko and Kopec, 1963). A small granule component, of uncertain identity and apparently previously unreported, was constantly discernible in the cytoplasm of cells in all preparations studied (Figs. 231, 233, 237). Evidence of the aggregations of basophilic material as rods or granules (Deane, 1946, Lagerstedt, 1949; Bernhard et al., 1952; Strenram, 1953, 1954; Finck, 1958; Baradwaj and Love, 1959; Colosi and Marsilii, 1960) was not found in living hepatocytes observed with phase-contrast microscopy.

CHAPTER IV

EXPERIMENTS ON THE INTRACELLULAR DISTRIBUTION OF CARCINOGENIC HYDROCARBON IN LIVING L-929 FIBROBLASTS AND HEPATIC CELLS

Introduction

The intracellular distribution of CHs has been studied in a wide variety of living vertebrate and invertebrate cell types, prepared from free-living cells or from whole tissues by cell dissociation (Graffi, 1939, 1940; Graffi and Maas, 1940; Günther, 1941/1942; Waddington and Goodhart, 1949; Richter, 1951, 1952a and b, 1955; Richter and Saini, 1960; Shires, 1962; Jones, 1962; Harvey et al., 1963). Consideration of the CH patterns in living cultured cells has been limited to material from chick heart primary explants (Richter, 1952a; Richter and Saini, 1960) and from several long-term culture strains (Richter and Saini, 1960; Harvey et al., 1963).

In this study, dissociated living hepatic parenchymal cells and cells from a tissue culture cell strain (Strain L-929 fibroblasts) were observed with the integrated phase-contrast and u-v-fluorescence microscopic procedure after exposure to 3:4 benzpyrene (BP). These two cell types not only represent contrasting in vitro systems but differ in

other ways. 1) Hepatic parenchymal cells have, normally, a low mitotic frequency (Bucher, 1963), while L-cells may double their total population in approximately four days of growth. 2) Liver cells and L-cells may be expected to differ in metabolic capacity with regard to polycyclic hydrocarbons. Hepatic parenchymal cells are the known sites of the apparently substrate-inducible enzyme, benzpyrene hydroxylase (Conney et al., 1957; Gelboin and Sokoloff, 1961; Acros et al., 1961; Wattenberg and Leong, 1962; Wattenberg et al., 1962; Loeb and Gelboin, 1963; Gelboin and Blackburn, 1962; Chang and Bond, 1964), while stromal fibroblasts of a number of organs have shown no evidence of possessing the enzyme (Wattenberg and Leong, 1962; Wattenberg et al., 1962). The oxidized hydrocarbon products of this enzymatic reaction (Conney et al., 1957; Harper, 1958; Acros et al., 1961; Falk et al., 1962) have a greenish blue fluorescence (Berenblum and Schoental, 1943; Berenblum et al., 1943; Doniach et al., 1943; Wattenberg et al., 1962; Wattenberg and Leong, 1962).

The expected absence of BP oxidative enzyme and the high mitotic activity of L-929 fibroblasts were two physiologic characteristics advantageous to exploratory investigation of several specific problems concerning the intracellular distribution of CH and non-CH in living cells. It was anticipated that, in L-cells growing on crystalline beds of BP or Py, the morphological status of unaltered hydrocarbon could be followed over the time course of exposure with/without further alterations in the culture conditions. Thus the following experiments were carried out: 1) a comparison of the

intracellular distribution of BP in cells grown on crystalline beds of BP with that in cells exposed to BP on glass slides just prior to observation, 2) a study of the intracellular distribution of pyrene in contrast with that of BP in fibroblasts grown in vitro on the hydrocarbon, 3) a study of the intracellular distribution of BP in L-cells at various stages in the mitotic cycle, 4) a study of the effects of simultaneous exposure of L-cells to BP and to prolonged irradiation with u-v light, 5) a study of the intracellular distribution of BP in cells growing on crystalline beds and concomitantly exposed to the co-carcinogen, croton oil.

Materials and Methods

The different procedures for exposing liver cell suspensions and L-929 fibroblast cultures to BP and Py have been previously described.

Irradiation with u-v light was done on in vitro slide preparations of L-cells grown on crystalline beds of BP. The slides were placed on the stage of the u-v-fluorescence microscope and a selected high power (990X) field was continuously irradiated with raised substage condenser for 10-20 minutes. The irradiated field was then observed with the integrated phase-contrast and u-v-fluorescence microscopic procedure. The control preparations for this experiment were unirradiated in vitro slide preparations.

In order to achieve the combined exposure of L-929 fibroblasts, suspensions of the cells were seeded onto crystalline beds of BP and grown for 24 hours. At this time, a 10 mm. square of autoclaved

standard blotting paper, saturated with the co-carcinogenic substance, croton oil, was added to the culture medium. Live fibroblasts were observed after one to four hours exposure to the combination of agents. In control, L-cell cultures were exposed to crystalline beds of BP only.

Observations

The Intracellular Distribution of Benzpyrene in Intact Parenchymal cells of the Rat Liver

The intracellular pattern of the fluorescent carcinogenic hydrocarbon included a differential accumulation within the hepatic cell cytoplasm. In relation to the general level of CH accumulation in the cytoplasmic matrix, the relatively highest concentrations of apparent BP was displayed by the presumptive Golgi complex, lipid droplets and the small granule component (Figs. 232, 234, 236, 238). Relatively little CH uptake could be discerned in the mitochondrial compliment of the living hepatic cells (Figs. 234 and 238). No demonstrable uptake of CH was shown by any of the nuclear structures, including the nuclear membrane, nucleolus, chromatin and karyolymph.

The color of intracellular fluorescence in the <u>in vitro</u> preparations was always observed to be light bluish. Control preparations were usually non-fluorescent. Occasionally, large masses of imperfectly dissociated material showed a light yellowish color when viewed under u-v light. However, isolated cells on these preparations were never found to be fluorescent, either by direct observation or photographically.

Experimental Studies on Living L-929 Fibroblasts Relative to the Intracellular Distribution of Carcinogenic and Non-Carcinogenic Hydrocarbons

The intracellular distribution of benzpyrene in cells exposed for variable periods of time. Living L-929 fibroblasts, exposed to benzpyrene for 24 to 72 hours and viewed with the integrated phase-contrast and u-v-fluorescence microscopic procedure, contained discretely compartmentalized intracellular apparent carcinogen. Where the cytoplasm was clear of inclusions, the cytoplasmic matrix throughout the cell showed demonstrable accumulation of BP (Figs. 2, 3, 5). The various types of cytoplasmic processes demonstrated an apparently slightly lower relative accumulation of BP because of their thinness (Figs. 2, 3, 5). Of the cytoplasmic inclusions, mitochondria, both rod-shaped (Fig. 2) and granular (Fig. 5), showed relatively lower concentrations of CH when favorably placed in thin cytoplasm. The granules aggregated in the juxtanuclear Golgi zones, as well as the peripheral granules of the dispersed form, displayed the relatively highest intracellular accumulations of BP (Fig. 9). All nuclear structures, including nuclear membrane, nucleolus, chromatin and karyolymph and nuclear fragments, displayed no demonstrable accumulations of CH in any cell observed (Figs. 2, 3, 5).

L-929 Fibroblasts, exposed to crystalline beds of BP on glass slides just prior to observation, showed the same general pattern of intracellular carcinogen as was found in those cells exposed to the CH for longer periods of time. The relatively highest concentrations of BP were found in the Golgi zones and Golgi granules, and no apparent

BP uptake was discernible in mitochondria (Figs. 9, 11). No demonstrable accumulation of CH was observable in any nuclear structure (Figs. 9, 11), while the intranuclear cytoplasmic tongues displayed an accumulation of BP comparable with that of the general cytoplasmic matrix (Fig. 11).

The intracellular distribution of benzpyrene in mitotic cells. Viewed under u-v-fluorescence microscopy, no demonstrable benzpyrene was found within the prophase nucleus (Fig. 15), within the chromosomal masses of metaphase (Figs. 16, 18) and the daughter chromosomes of anaphase (Figs. 20, 23, 26) or within telophase karyomeres and developing nuclei (Figs. 15, 29). L-fibroblasts grown on crystalline beds of benzpyrene and observed at the end of the log growth phase displayed no intranuclear accumulations of BP, showing that no invasion by BP into chromosomal structures occurred during their term without nuclear membranes or during the phases of karyomeric aggregation and nuclear reconstruction (Fig. 29). Nuclear membranes occurring in prophase and telophase both accumulated relatively higher concentrations of BP.

The early anaphase interzonal spindle region displayed the same lack of affinity for carcinogens as the daughter chromosomes (Fig. 20), but as anaphase proceeded the interzonal region showed increased uptake of BP (Fig. 26) until apparent interzonal concentrations were similar to those in the general cytoplasmic matrix in early telophase (Fig. 23). No special compartmentalization of BP was noted in the presumptive polar cytoplasmic area of metaphase (Figs. 16, 18) or anaphase (Figs. 20, 23, 26) compared with non-polar regions.

BP accumulations were relatively highest in Golgi granules at all stages in the mitotic cycle (Figs. 15, 16, 18, 20, 23, 26, 29) even when they occurred at the edge of, or in, the interzonal spindle area. Relatively low demonstrable BP uptake occurred in mitochondria throughout mitosis (Figs. 15, 16, 29) and in the interna of cytoplasmic surface blebs (Figs. 16, 17, 23, 29). Cytoplasmic rims of the blebs, the fine cytoplasmic processes (Figs. 16, 18) and the telophase equatorial plate region all displayed carcinogen accumulations comparable with those in the general cytoplasmic matrix.

The intracellular distribution of pyrene in living cells. Pyrene has been shown to be a non-carcinogenic, polycyclic, fluorescent hydrocarbon, a close structural relative to benzpyrene. The solubility of pyrene in water is somewhat greater than BP (Rodd, 1956). Living L-cells, mounted in complete nutrient media on crystalline beds of pyrene, showed very low levels of pyrene uptake, even when the cells were immediately adjacent to pyrene crystals. U-V-fluorescence microscopic observation of L-cells after incubation of the living-cell preparations at 37°C for 15-20 minutes encountered dimly fluorescent fibroblasts, photomicrographs of which were unobtainable even with prohibitively long exposure times (one minute and greater). Study of the intracellular distribution of pyrene made on fibroblasts seeded on crystalline beds of pyrene and grown 24-36 hours prior to observation showed easily observable levels of fluorescence.

L-cells grown on pyrene crystals showed cytomorphologic alterations not found in cells grown on benzpyrene for similar periods of

time. Various sizes of dark granules, ranging up to approximately 1.2 micra, appeared in the central and intermediate cytoplasmic regions, the larger granules usually situated peripherally (Figs. 31, 32, 34, and 35). The larger granules generally showed differentiated light and dark regions (Figs. 31 and 34), while the smaller granules were uniformly dark (Figs. 31, 32, 34, and 35). Apparent fusion of some granules was noted in some cells (Figs. 31 and 32). Similar granules have been reported in the cytoplasm of avian fibroblasts exposed to high concentrations of colchicine in vitro (Wilson, 1962).

Nuclei of pyrene-exposed fibroblasts contained variable-sized nucleoli, heterochromatin and nuclear membranes similar in appearance to BP-treated cells (Figs. 31, 32, 34, and 35).

Under the u-v-fluorescence microscope, no cytoplasmic organelle displayed pyrene accumulations demonstrably greater than those of the general cytoplasmic matrix (Figs. 33 and 36). The cytoplasmic granules, throughout their size range, appeared to show relatively little uptake of apparent pyrene (Figs. 33 and 36). All nuclear structures failed to demonstrate any pyrene accumulation (Figs. 33 and 36). No autofluorescence of cells was observed on any of the control preparations.

Effects of u-v light on the intracellular distribution of benzpyrene. It is known that the u-v irradiation of CH-containing cells produces alterations at biochemical, cytophysiologic and cytomorphologic levels. Rapidly deleterious photodynamic effects of u-v light have been reported on unicellular organisms (Mottram and

Doniach, 1938; Doniach, 1939; Hollaender, 1939; Calcutt, 1954; Epstein and Zucca, 1962; Epstein, 1963; Santamaria, 1963; Epstein et al., 1964). U-V-photo-dynamic damage to CH-containing metazoan cells has been localized cytologically (Lewis, 1935; Earle et al., 1943) and physiologically (Blum, 1959; Santamaria, 1963). Under prolonged exposure to u-v-light, photoreaction has been reported between BP and deoxyribonucleic acid (DNA), various nucleotides and various pyrimidines and purines (Rice, 1964; Ts'o and Lu, 1964; Ts'o et al, 1964).

The possibility of these photodynamic effects influencing the apparent intracellular distribution of CH was considered by Richter and Saini (1960), but no effects were found. In an extension of this consideration, fields of L-cells growing on crystalline beds of BP, were irradiated continuously 6-10 minutes on the stage of the u-v-fluorescence microscope and the observed intracellular pattern of BP compared with that found in cells irradiated for shorter periods of time.

Phase-contrast microscopic observations. L-cells exposed to prolonged u-v-irradiation showed surface blebbing but little other manifest alteration (Fig. 12). Blebbing by irradiated cells has been reported by Lewis (1935) and Calcutt (1954). In mitotic cells, signs of derangement of chromosomes on metaphase plates were noted (Fig. 12) (Blum, 1959).

U-V fluorescent microscopic observations. The apparent intracellular distribution of BP in cells exposed to long-term irradiation (Fig. 13) appeared similar to that in cells exposed to u-v light only for photographic and observational purposes (less than one minute).

All nuclear and chromosomal structures showed no evidence of carcinogen uptake (Fig. 13). The Golgi granules displayed the relatively highest accumulations of BP in the cell (Fig. 13). Thus there is no evidence that u-v light influences the apparent intracellular distribution of BP.

Intracellular distribution of benzpyrene after exposure to croton oil. In a two-step hypothetical mechanism of carcinogenesis, tissues must successively come into contact with a chemical initiator, such as BP, followed by a promoting cocarcinogenic agent (Bereblum, 1951). Of a wide variety of cocarcinogenic agents, croton oil was one of the first discovered (Bereblum, 1941). When croton oil was added to medium in which fibroblasts were growing on BP beds, surface blebs appeared in all cells within 10-20 minutes. (Fig. 38). Otherwise no major cytomophologic changes were observed.

Under u-v-fluorescence microscopy the pattern of BP accumulation within the cells was essentially similar to that in cells not exposed to the cocarcinogen, except for distinct relatively high accumulations of BP in the nuclear membrane region (Fig. 37). No apparent BP uptake was demonstrable in other nuclear structures, including nucleolus and the general karyoplasmic region (Fig. 37). Chromosomal material also displayed no affinity for the CH, as was the case for the interzonal spindle region (Fig. 37). The relatively highest accumulations of carcinogen in cells exposed to croton oil appeared to occur in the dark Golgi granules (Fig. 37).

Discussion

Living Interphase Cells

The intracellular distribution of BP in L-929 fibroblasts and hepatic parenchymal cells was essentially similar regarding comparable structures in both cell types. The various forms of the mitochondrial component of fibroblasts and liver cells showed no apparent BP uptake. Elements of the Golgi complex of both cell types displayed concentrations of the CH which were comparatively greater than those in the general cytoplasmic matrix. No demonstrable accumulation of BP was apparent in the nuclear structures of fibroblasts and liver cells, such as the nucleolus, chromatin and karyolymph.

The distribution of BP relative to mitochondria, Golgi elements, cytoplasmic matrix and the various nuclear structures of Strain L-929 fibroblasts and hepatic parenchymal cells is similar to the distribution of BP in comparable cytologic structures of all nucleated cell types previously studied under physiologic conditions (Graffi and Maas, 1940; Günther, 1941/1942; Richter, 1951, 1952a and b, 1955; Richter and Saini, 1960; Shires, 1962; Jones, 1962; Harvey et al., 1963). These similarities in the relative intracellular distribution of CH in living cells constitutes a standard pattern, against which all experimental results may be compared.

Mitotic L-929 fibroblasts. By growing fibroblasts on crystalline beds of BP over a period of several days, the intracellular distribution of CH could be studied as it came to exist in different stages of the mitotic cycle. The results of this study involved three mitotic structures: the nuclear membrane, the chromosomal material

and the interzonal spindle.

The nuclear membrane. A demonstrable accumulation of BP was found in the region of the nuclear membrane in early prophase and in the newly reconstructed membrane of telophase, but not in the nuclear membrane of interphase. Since these mitotic membranes are involved in dispersion or aggregation at these respective periods of mitosis, their biochemical content may be expected to vary from that in interphase. Richter and Saini (1960) found evidence of relatively higher BP concentration in the nuclear membranes of mouse primary spermatocytes and spermatids, cells about to enter or leave meiosis. Two or three other reports of discernible nuclear membrane uptakes of CH have involved cells with high mitotic frequency: ascites tumor cells (Graffi, 1939, 1940) and basophilic erythroblasts (Jones, 1962). The third report was that by Shires (1962) of demonstrable nuclear membrane accumulations of BP in epididymal epithelial cells. When all the facts concerning the accumulation of CH in the nuclear membrane at different phases of the growth cycle are considered together, they show an apparently physiologically-linked variability in the affinity of that structure for the carcinogen. No previous observations regarding physiologically-linked variations in the intracellular pattern of CH have apparenly been reported.

Mitotic chromosomal material. Chromosomal material in all stages of mitosis showed no demonstrable affinity for BP. Waddington and Goodhart (1949), working with spreads of Drosophila salivary gland chromosomes, reported the same results, as did Berg (1951/1952), in studies on fixed and frozen-sectioned root tips, and Richter and

Saini (1960) for mouse germinal epithelial cells. Several points about this lack of chromosomal affinity for BP have been previously emphasized:

1) It has been repeatedly reported, although never directly for the Stain L-929 fibroblasts, that metaphase and anaphase chromosomes are not surrounded by any membrane (Moses, 1958; Barer et al., 1958; Waley et al., 1960; Harris, 1961). The observation that no demonstrable BP uptake occurred in anaphase and metaphase chromosomes, as well as in interphase, prophase and telophase nuclei, suggests that the nuclear membrane plays no role in determining the characteristics of BP uptake by intranuclear structures.

2) The ploidy status of chromosomes appears an unimportant factor in their characteristic lack of affinity for CHs. The same lack of affinity appears in the haploid chromosomes of germinal epithelium (Richter and Saini, 1960), in polytene salivary gland chromosomes (Waddington and Goodhart, 1949) and in the aneuploid conditions of Strain L-929 chromosomes.

3) The lack of affinity for BP by chromosomal material is constant at all stages of mitosis. As the chromatid pairs undergo metakinesis, as the daughter chromosomes split and move polarward and as the karyomeres swell, aggregate and differentiate, no demonstrable BP uptake was found in any of the chromosomal structures. These stages in mitosis are known to represent different states of cytophysiology, both within the chromosomal material and in the surrounding protoplasm (Mazia, 1960).

4) No evidence was found for any incorporation of apparent BP into post-mitotic nuclei, as might have been expected according to the hypotheses of Mottram (1945), Frei and Ritchie (1964) and Boyland (1964). There was demonstrable BP accumulation by perichromosomal protoplasm in metaphase plates and between anaphase chromosomes, but dispersion of this protoplasm appeared to occur during the telophase reformation, leaving the interior of the telophase nucleus without apparent accumulations of BP.

The interzonal spindle. The interzonal spindle represents, after the nuclear membrane, a second structure which displays a variable affinity for BP. Early anaphase spindles showed no demonstrable BP accumulation, except in a few Golgi granules caught in the spindle substance. Later anaphase interzonal spindles show accumulations of BP which appear to increase to levels comparable with those of the cytoplasmic matrix as anaphase proceeds into telophase. As reported by Boss (1955) and confirmed with Gallocyanin-Chrom Alum staining, late anaphase interzonal spindles are associated with a marked increase in nucleic acid content. Thus the advent of nucleoproteins on the interzonal spindle occurs at approximately the same time as the advent of an increase in apparent BP uptake.

Environmental Factors in the Intracellular Distribution of CH in living L-929 Fibroblasts in Vitro

Neither length of exposure to BP, irradiation with u-v light or exposure to the carcinogen, croton oil, was found generally to alter the pattern of intracellular BP.

L-fibroblasts grown on crystalline beds of BP several days compared with those exposed just before observation showed identical intracellular distribution of the CH.

L-cells grown on BP and irradiated with u-v light for prolonged periods of time showed surface blebbing, but no signs of any alteration in the basic pattern of intracellular CH. These results contribute to a measure of confidence that the integrated u-v fluorescence and phase-contrast microscopic procedure does not alter the intracellular distribution of CH.

Simultaneous exposure of cells to BP and croton oil also produced surface blebbing, but no other cytomorphologic changes were observed. The appearence of a distinct nuclear membrane uptake of BP in some fibroblasts was the only exception to an intracellular distribution of BP which was otherwise similar to that in untreated interphase and mitotic cells. The meaning of the croton oil-associated uptake of BP by the nuclear membrane in comparison with related observations of BP uptake in the nuclear membranes of other cell types (Graffi, 1939, 1940; Richter and Saini, 1960; Shires, 1962; Jones, 1962) is not apparent.

The Intracellular Distribution of Pyrene in Living L-929 Fibroblasts

Study of the intracellular distribution of pyrene in living L-fibroblasts was hampered by the apparently low affinity of protoplasm for the non-CH, in comparison with the CH, BP. Study of cells exposed to Py for short periods of time resulted in the appearance of

cytoplasmic inclusions not observed either in unexposed cultures or in cultures exposed to the CH, BP, for similar periods of time.

Repeated observations at the light and ultrastructural levels have found cytoplasmic alterations, especially in the mitochondrial component, in cells exposed to polycyclic CHs (Stewart, 1939; Orr; 1939, 1963; Porter and Bruni, 1959; Friedlander and Bunggeli, 1959; Setälä et al., 1960; Burt et al., 1961; Mylius, 1962; Scarpeli and von Haam, 1963) and to other types of chemical carcinogens (Arcos et al., 1961; Endo and Kyushu, 1963; Mikata and Luse, 1964; LaFontaine and Allard, 1964). No reports of similar changes have appeared for cells treated with non-CHs, although the appearance of special cytoplasmic inclusions has been reported by electron microscopists in cells treated by such cocarcinogens as urethane (Frei and Sheldon, 1961; Klärner and Gieseking, 1963). Without correlative ultrastructural data concerning the cytoplasmic alterations in L-cells exposed to Py, it is not known how they compare with those induced by CHs and cocarcinogens.

The occurrence of these cytoplasmic inclusions after exposure of L-929 fibroblasts to Py makes the interpretation of the apparent intracellular distribution of Py as viewed with integrated phase-contrast and u-v-fluorescence microscopy difficult. One distinct feature of the intracellular pattern of Py is the absence of apparent Py from any nuclear structure. Pyrene shares this characteristic of distribution with the carcinogen, BP, and with BP in the presence of the cocarcinogen, croton oil.

CHAPTER V

STUDIES ON THE INFLUENCE OF HISTOLOGIC PROCESSING AGENTS ON THE INTRACELLULAR DISTRIBUTION OF CH AND NON-CH

Introduction

In general, this investigation concerns the intracellular distribution of BP and Py in L-929 fibroblasts as influenced 1) by a variety of fixatives and fixation methods and 2) by certain post-fixation processing methods.

The experimental studies fall into eight categories.

The first category of study concerns the comparative intracellular distribution of BP following fixation with the individual or combined components of a conventional complex fixative, Zenker's fluid (a mixture of acetic acid, potassium dichromate and mercuric chloride).

The second category of study concerns comparative examination of the intracellular distribution of CH in cells fixed with a variety of multi-agent fixatives.

The third category is a study of the comparative intracellular distribution of BP following processing with solutions containing formaldehyde as the only fixing agent. It includes the following three

facets: 1) a comparison of the effects of four different solutions of formaldehyde in various concentrations and with different salt and buffer solutions, 2) a study of the fixation of cells grown on BP in contrast with those not exposed to BP until after fixation, and 3) a study of the effects of the length of fixation time.

The fourth category of study involves consideration of the effects of fixation with six solutions containing single fixing agents on the intracellular distribution of BP.

The fifth category of study is a comparative investigation of the intracellular distribution of the non-CH, Py, in cells fixed with a variety of solutions containing single fixing agents which previously had been studied relative to the intracellular distribution of the CH, BP.

The sixth and seventh categories of study are related to the influence of fixation of cells with vapors and with freezing on the intracellular distribution of CH, respectively.

The eight category deals with an investigation of the alterations in the intracellular distribution of BP in cells exposed to two post-fixation procedures: the removal of mercury from cells fixed with $HgCl_2$-containing fixatives and the dehydration and clearing of fixed cells.

Materials and Methods

The study of the influence of conventional histologic processing agents on the intracellular distribution of CH and non-CH was limited to the L-929 fibroblast as test object. The agents under consideration were a large variety of conventional chemical fixatives, freezing and agents involved in such post-fixation procedures as dehydration and clearing or mercury removal. Exposure of cells to CH or non-CH was carried out either before fixation (by growing L-fibroblasts on crystalline beds of BP or Py) or after fixation (by mounting coverslips bearing the treated cells on beds of BP on glass slides). All preparations were mounted in water with the coverslips sealed by ringing with beeswax, and examined with the integrated phase-contrast and u-v-fluorescence microscopic methods previously described.

Fixatives and Technical Processing Agents Tested

The following chemical fixatives and the solutions in which they were utilized are here listed.

1. Four different solutions containing only the agent formaldehyde (HCHO) were tested:
   a) an aqueous solution of 40% HCHO,
   b) 4% HCHO in 0.1M phosphate buffer at pH 7.4 (Lillie, 1948)(Lillies-HCHO),
   c) 4% HCHO in an aqueous 10% calcium chloride solution (Baker, 1946)($CaCl_2$-HCHO),

d) 4% HCHO in saturated aqueous solution of calcium carbonate (approximately 0.13-0.14%)(Lillie, 1948)($CaCO_3$-HCHO);

2. Potassium dichromate in an aqueous 2.5% solution (Baker, 1960);
3. 5% aqueous acetic acid (HOAc)(Baker, 1960);
4. a saturated solution of mercuric chloride ($HgCl_2$)(approximately 5-6%)(Baker, 1960);
5. 95% aqueous ethanol (EtOH)(Baker, 1960);
6. 2% osmium tetroxide in an aqueous veronal acetate buffer at pH 7.4 made according to Palade, 1952 (OsO_4);
7. 4% glutaraldehyde in an aqueous 0.1M phosphate buffer at pH 7.2 (Sabatini et al., 1963);
8. a mixture of 2.5% potassium dichromate, 5% $HgCl_2$ and 5% HOAc (Zenker's fluid, used after being freshly made or after three months aging) made according to Cowdry (1948);
9. a mixture of saturated picric acid, 4% HCHO and 5% HOAc in the ratio of 25:5:1 made according to Cowdry, 1948 (Bouin's fluid);
10. a mixture of 2.5% potassium dichromate and 5% $HgCl_2$ after Cowdry, 1948 (Zenker's stock);
11. a mixture of equal volumes of 40% HCHO and 1% chromic acid after Chayen et al., 1957 (Lewitsky's fluid);
12. a mixture of 5% HOAc and 2.5% potassium dichromate after Casselman, 1955 (acidified dichromate).

Fixation with these agents was obtained by immersing coverslip cultures of L-929 fibroblasts in fixing solutions contained in Columbia staining dishes. An alternate method in the case of one HCHO-fixative was exposure of coverslip cultures to fixing vapors. Fixation times for the simple HCHO fixatives ranged from five minutes to three days; for Zenker's fluid and various Zenker's components (e.g. HOAc, $HgCl_2$, dichromate, acidified dichromate and Zenker's stock), one to two hours; for EtOH, 5 minutes to two hours; for OsO_4, two to three hours; for Lewitsky's fluid, 10 minutes to two hours; and for Bouin's fluid, two to 20 hours.

Procedure Involving Freezing of Cells

Freezing was combined with chemical fixation in this study. Freezing was carried out directly on L-fibroblasts growing on coverslips by one slow and three fast freezing procedures.

Slow freezing was accomplished by placing the coverslip cultures on a block of dry ice (solid carbon dioxide temperature approximately -78^0C) for approximately five minutes.

Rapid freezing was done by 1) immersing the coverslip cultures directly in liquid nitrogen (-196^0C)(after Bloom et al., 1954), 2) immersion of the culture vessel, containing the coverslip cultures, in liquid nitrogen, or 3) immersion of the coverslip cultures in isopentane vapor cooled to -196^0C by liquid nitrogen (after Bloom et al., 1954).

After freezing, the coverslip cultures were transferred to

Lillie's-HCHO and fixed one hour at room temperature.

Post-Fixation Procedures Tested

Two post-fixation technical sequences were evaluated: a) mercury deletion procedure and b) a dehydration and clearing procedure.

The mercury deletion process consisted of treating cells fixed with $HgCl_2$-containing fixatives, such as Zenker's fluid, for 5-10 minutes with Lugol's iodide-iodate solution followed by treatment with a 5% solution of sodium thiosulfate (Gridley, 1960). The preparation was then washed and mounted and observed in water with or without exposure to BP.

The dehydration and clearing process was evaluated on L-929 fibroblasts fixed on $CaCl_2$-HCHO, dehydrated in 35%, 50%, 70%, 90% and 95% and 100% EtOH and cleared in 50:50 EtOH: toluene and 100% toluene. The preparation was rehydrated by the same steps in reverse, mounted and observed in water. The time at each step was one minute.

Observations

The Influence of Zenker's Fixative and Its Components on the Intracellular Distribution Carcinogenic Hydrocarbon

Effects of fresh and aged complete Zenker's fluid. In comparison with the live cell structure, structural alterations were similar in cells fixed by fresh or aged (three months old) Zenker's Fluid.

Fixation of L-929 fibroblasts' cytoplasm with fresh or aged Zenker's fluid produced a coarse network of precipitated material,

visible in detail in peripheral cytoplasmic regions (Figs. 73, 80, 84, 87). Numerous strands and irregular masses of varying degrees of refractility filled the central cytoplasmic region (Figs. 73, 80, 84, 87). Mitochondria were not generally discernible in the cytoplasmic precipitate (Strangeways and Canti, 1927) (Figs. 73, 80, 84, 87). A few vacuolar structures were detected in the cytoplasm (Figs. 73, 84). Interzonal spindle fibers, not visible in the spindles of living fibroblasts, were distinct structures after Zenker's fluid fixation (Fig. 81, 83), but no astral structures were found.

The chromatin material in nuclei of cells fixed with either fresh or aged Zenker's fluid was finely precipitated and more discrete than in living cells. Both chromatin and nucleoli tended to be variably refractile, often to the detriment of observing intranuclear structure (Figs. 73, 75, 77, 80, 84, 87). Mitotic chromosomal material was phase-light (compared with its phase-dark appearance in living mitotic cells) (Figs. 77, 80, 81, 83).

The pattern of intracellular distribution of BP was essentially similar in cells fixed with either fresh or aged Zenker's fluid. Cytoplasmic vacuoles in the cytoplasm of these cells showed no demonstrable BP uptake (Figs. 73-74, 77-80, 84-85), while Golgi zones, Golgi granules and frank lipid droplets showed relatively higher accumulations of BP than those in the general cytoplasmic matrix (Figs. 73-87). In all cells studied, demonstrable accumulation of CH in nuclear chromatin, nucleoli, and the nuclear membrane was not found (Figs. 73-87). Chromosomal material in metaphase, anaphase and telophase also showed

no demonstrable affinity for the carcinogen (Figs. 77-83). The relative affinity for BP of the interzonal spindle in later stages of anaphase and early telophase was comparable with that by the general cytoplasmic matrix for the CH (Figs. 78-83).

The pattern of intracellular distribution of CH in cells fixed with either fresh or aged Zenker's fluid was similar to the pattern in living L-cells, except for the new structures, such as cytoplasmic vacuoles and frank lipid droplets, which formed as a result of the action of the fixing agent on the cells.

Effects of fixation by the individual components of Zenker's fluid. 2.5% Potassium Dichromate. L-929 fibroblasts fixed in 2.5% dichromate were greatly altered in their phase-contrast microscopic appearance in comparison with living cells. The cytoplasm of these cells was generally full of vacuoles, and all other cytoplasmic structures were packed in between these vacuoles (Fig. 99). Identification of specific structures under these conditions was difficult. The nucleus of cells fixed with dichromate was filled with various sizes of amorphic masses, embedded in a relatively phase-dark karyoplasmic matrix (Fig. 99). No nucleoli were discernable (Fig. 99) (Strangeways and Canti, 1927).

The nuclei of dichromate-fixed cells showed no convincing evidence of BP accumulation by nuclear membrane or the general intranuclear region (Figs. 99, 100). No demonstrable accumulation of BP was apparent in cytoplasmic vacuoles (Fig. 99, 100). The distribution of BP relative to other cytoplasmic structures was not possible to

determine because of the adverse conditions within the cytoplasm. The pattern of intracellular distribution relative to the general nuclear region was identical with that in similar regions of living cell (Figs. 1-11).

Mercuric Chloride. The cytoplasm of cells fixed with 5-6% $HgCl_2$ was filled with a fine precipitate, some variably sized dark granules (appt to 0.6 micra in diameter), occasional vacuoles and some frank lipid droplets (Figs. 104, 106). None of these structures were normally found in the cytoplasm of living fibroblasts (Figs. 1, 4, 6, 7, 8, 10). The nuclei of $HgCl_2$-fixed cells contained a finely precipitated chromatin material and refractile nucleoli (Figs. 104, 106) (Baker and Luke, 1963).

Observation of $HgCl_2$-fixed cell with integrated phase-contrast and u-v-fluorescence microscopy showed no demonstrable accumulation of the fluorescent CH by any nuclear structure, including the nuclear membrane, chromatin and nucleoli (Figs. 103-106). In the cytoplasm, the frank lipid droplets displayed the highest apparent concentrations of BP, while cytoplasmic vacuoles apparently showed the lowest BP uptake, compared with that by the general cytoplasmic matrix (Fig. 103-106).

The intracellular distribution of BP in cells fixed with $HgCl_2$ was identical to that in living cells in regard to structures preserved by the fixative.

Acetic Acid. 5% HOAc, when applied to L-929 fibroblasts, preserved little more than the general outline of the original cell. Nuclear material appeared completely dispersed (Fig. 98). Cytoplasmic

regions of the cell were reduced to an aggregation of granules and strands of material and frank lipid droplets (Fig. 98) (Baker, 1957).

Benzyprene uptake by these badly distorted cells was demonstrable in the general material of the disorganized cytoplasm (Figs. 97-98).

No meaningful interpretation of the intracellular distribution of CH in cells fixed with 5% HOAc is possible due to the general destruction of cell structure.

Effects of fixation by various combinations of individual Zenker components on intracellular distribution of BP. 5% HOAc and 2.5% Potassium Dichromate. Alteration in the phase-contrast microscopic appearance of L-929 fibroblasts fixed with acidified dichromate solution (similar to a solution of chromic acid, Casselman, 1955), was marked. Both the nuclei and cytoplasm of cells fixed with the acidified dichromate solution were filled with refractile material which tended to obscure surrounding structures (Fig. 94, 96). Lipid droplets, inclusions rarely encountered in living cells (Figs. 1, 4, 6, 7, 8- 10), were frequent (Figs. 94, 96).

Cells fixed with acidified dichromate and exposed to BP showed a relatively low intensity of general cellular fluorescence. Markedly higher accumulations of apparent BP were localized in lipid droplets (Fig. 94-98), relative to the BP uptake by the general cytoplasmic matrix. No demonstrable accumulation of BP was discernible in any nuclear structure (Figs. 94-96).

In a comparison of cytologic structures present in both living

and fixed cells, the pattern of intracellular BP was preserved relative to nuclear structures.

2.5% Potassium Dichromate and 5-6% Mercury Chloride. Fixation with this mixture of dichromate and $HgCl_2$ (Zenker's stock) effected a preservation of fibroblast structure similar to that of the living cells (Figs. 90, 93, vs. Figs. 1, 4, 6, 7, 8, 10) (Cowdry, 1948). Occasional fine vacuolation and the occasional appearance of lipid droplets represented the only major cytoplasmic difference between the live cell appearance (Fig. 90, 93). Within the nucleus, chromatin occurred as a fine precipitate (Fig. 90, 93).

No demonstrable accumulation of the CH could be found in any nuclear structure in cells fixed with Zenker's stock (Figs. 90-93). In the cytoplasm, the relatively highest accumulations of BP were localized in Golgi granules and lipid droplets (Figs. 90-93), compared with the general accumulation in the cytoplasmic matrix. No evidence of BP uptake by mitochondria or cytoplasmic vacuoles was found (Figs. 90-93).

This pattern of intracellular BP closely resembled that in living cells (Figs. 1-11). Differences were due to the occurrence of fixation-produced structures such as cytoplasmic vacuoles and lipid droplets.

Studies on the Influence of Fixatives Containing Several Fixing Agents on the Patterns of Intracellular BP

The effects of Zenker's multi-agent fixative on the patterns of intracellular BP, and the effects of acidified dichromate and

Zenker's stock solution have been presented in earlier sections. The effects of two additional multi-agent fixatives were studies: Bouin's fluid and Lewitsky's fluid.

Bouin's Fluid. Cells fixed in Bouin's fluid for 1 hour and then exposed to crystalline beds of BP were found to contain a coarse cytoplasmic and nucleoplasmic granularity which tended to obscure the identity of various structures (Fig. 165) (Strangeways and Canti, 1927; Buchsbaum, 1948). This granularity was neither characteristic of HCHO-containing fixatives (viz. 39-58) or of cells fixed in HOAc-containing agents (Figs. 98-99). A tendency of material to precipitate in the region of the nuclear membrane was frequently found (Fig. 195), making the nuclear membrane appear very thick. Within the nucleus, nucleolar structures were discernible (Fig. 165).

When observed with u-v-fluorescence microscopy, no nuclear structure, including the thickened nuclear membrane, showed any demonstrable BP uptake (Fig. 165-166). The differential uptake in the cytoplasm of cells fixed with Bouin's fluid involved relatively higher accumulations of BP in Golgi granules and the Golgi zone, while concentrations of CH, relatively lower than those in the general cytoplasmic matrix, occurred in some unidentifiable cytoplasmic granules (Figs. 165-166).

Lewitsky's Fluid. This complex dichromate-HCHO mixture has been primarily a botanical fixative, but was reported by Chayen et al. (1957) to fix mammalian cells in such a fashion that BP uptake by nucleoli occurred from Berg's BP-caffeine solution (Berg, 1951-1952).

Cells fixed for one hour in Lewitsky's fluid showed a highly refractile nucleus and cytoplasm (Fig. 119), characteristic of cells fixed in acidified dichromate solutions (Figs. 94-96). Only in the peripheral cytoplasmic regions could distinct structures, such as lipid droplets and vacuolar formations, be discerned (Fig. 119), although the nuclear membrane was seen with some degree of clarity (Fig. 119). Under the u-v-fluorescence microscope, no demonstrable uptake of BP was discernible in any nuclear structure, including the nucleolus (Figs. 119-120). The highest BP uptake in the cytoplasm was, in comparison with that by the general cytoplasmic matrix, in Golgi granules (Fig. 119-120).

The Influence of Fixative Containing Formaldehyde as the Injective Agent on the Intracellular Distribution of Carcinogens

Effects on cell structure. The several HCHO-containing fixatives produced characteristic alterations of the general live-cell structure.

40% HCHO-fixation was accompanied by the greatest alteration in live-cell structure (Figs. 1, 4, 6, 7, 10 vs. Fig. 44). Cytoplasmic alterations observed were the appearance of some fine granularity, moderate vacuolation, appearance of frank lipid droplets and increased obscurement of mitochondria (Fig. 44) (Buchsbaum, 1948; Crawford and Barer, 1951). Nuclear contents were all highly refractile, and most structural detail was obscured (Fig. 44).

$CaCO_3$-HCHO-fixation was accompanied by the formation of vesicles which formed and broke away from the cell surface as independent

structures and by some intracytoplasmic vacuoles (Fig. 41). Within the nucleus, the absence of resolution of chromatin structure was noticeable (Fig. 44).

$CaCl_2$-HCHO-fixed cells were only altered in relation to live-cell structure in the loss of distinctly resolvable chromatin structure within the nuclei (Figs. 45, 48, 49). Mitotic chromosomes also showed a lack of discrete structure (Fig. 51).

L-929 fibroblasts fixed in Lillie's HCHO also showed a loss of detailed chromatin structure (Figs. 52, 54, 55, 58). The occurrence of frank lipid droplets and a small amount of vacuolation was variable (Figs. 54, 55).

The pH of the fixatives tested ranged from 3.5 to 7.4 as previously noted. While the pH at which fixation proceeds has been reported to effect structural change (Strangeways and Canti, 1927; Petrunkevitch and Pickford, 1936; Buchsbaum, 1948; Crawford and Barer, 1951), the morphologic features noted here to characterize the different HCHO-containing fixatives cannot be specifically related to a pH effect; this experiment was not designed to study pH effects.

The duration of fixation by the different HCHO-containing agents ranged from five minutes to three days. The length of time for fixation had no discernible specific effect on the structure of the cells (Figs. 52, 54, 55, 58).

Intracellular BP distribution. The patterns of BP distribution in cells fixed by the different HCHO-containing fixatives were similar to each other (Figs. 41-58) and to those in living cells (Figs. 1-11).

As in the living cell, BP was demonstrably accumulated in Golgi zones and Golgi granules, and to a comparatively lesser degree in the general cytoplasmic matrix, but no apparent BP uptake was consistently found in any nuclear structure, in mitochondria, in mitotic chromosomes or interzonal spindles (Figs. 41-58). To the extent that HCHO-fixation effected the formation of structures not normally present in living cells, the HCHO-fixed cells showed some differences in the distribution of BP. Cytoplasmic vacuoles and the interna of vesicles showed no evident accumulation of BP (Figs. 41-49, 55-56), while frank lipid droplets and the vesicle rims showed distinct concentrations of apparent BP.

The duration of HCHO-fixation had no notable effect on the pattern of intracellular BP distribution (Figs. 52-58).

None of the various HCHO-containing fixatives used in this study had any demonstrable effect on the intracellular BP distribution, whether fixation was done after or before exposure to the CH (Figs. 41-44, 47-49, 55-58, Figs. 45-46).

Consideration of the Effect on Intracellular Distribution of BP by Various Fixatives Containing One Active Agent

Of the single-agent fixatives used in routine microtechnique and under investigation for their effect on the intracellular patterns of BP uptake in this chapter, four have been previously considered: HOAc, dichromate, HCHO and $HgCl_2$. Three additional single-agent fixatives were also examined for their influence on intracellular BP: EtOH, OsO_4 and glutaraldehyde. The latter two single-agent fixatives

are routinely used in electron microscopic fixation procedures.

95% Ethanol. The cytoplasm of L-cells fixed with 95% EtOH was filled with irregularly coarse, flocculated material (Figs. 109, 111, 112, 114, 116) (Strangeways and Canti, 1927; Buchsbaum, 1948). Among this cytoplasmic precipitate, some vacuolar structures were discernible but no named organelle could be detected (Figs. 109 and 112). The nuclei of EtOH-fixed cells contained a precipitate of varying degrees of fineness, one to several compact refractile nucleoli and a distinct nuclear membrane (Figs. 109, 111, 112, 114, 116). Evidence for the reportedly marked shrinkage of EtOH-fixed cells (Strangeways and Canti, 1927; Buchsbaum, 1948; Ross, 1953; Baker, 1960; Pearse, 1960) was not obtained in preparations fixed for one hour, but cells fixed for shorter periods of time displayed apparently increased nucleocytoplasmic ratios and nuclei whose general karyoplasm was less distinctly flocculated (Figs. 114, 166). It was not possible to decide if the nucleus had swollen in diameter or if the cytoplasm had shrunk. In general, the structural appearance of cells fixed in EtOH was markedly dissimilar to that of live-cells (Figs. 1, 5, 6, 7, 8, 10).

EtOH-fixed cells showed slightly higher accumulations of BP in the central cytoplasmic regions (Figs. 110, 115). Almost no specific cellular structures were observed to show accumulation of BP greater than that by the general cytoplasmic matrix. The general accumulation of BP by the cytoplasm was apparently low, and a well-defined differential uptake of BP was not observed, except relative

to cytoplasmic vacuoles which showed no apparent BP uptake (Figs. 110, 113, 115). In the nucleus, a discernible accumulation of BP by nucleoli in cells fixed for one hour was encountered (Figs. 110, 113). All other structures and regions of the EtOH-fixed nucleus displayed no demonstrable affinity for BP. (Figs. 110, 113, 115). Nucleolar uptake of BP was never encountered in living cells (Figs. 1-11).

Osmium Tetroxide. Solutions of OsO_4 have been well known for their fine preservation of cells, including fibroblasts *in vitro* (Lewis, 1917; Strangeways and Canti, 1927; Porter *et al.*, 1945; Buchsbaum, 1948; Crawford and Barer, 1951; Palade, 1952; Porter and Kallman, 1952, 1953; Frédéric, 1956; Baker 1960). In L-fibroblasts, mitochondria, Golgi granules, the structures of the Golgi zone, nucleoli and the nuclear membrane were blackened by 1 hour's fixation in Palade's buffered OsO_4 (1952), while the cytoplasmic matrix and karyoplasm were slightly darkened (Fig. 123). The close correlation between reduction of OsO_4 and lipid concentration at specific cell sites (Baker, 1960) has been shown to be due to the presence of ethylenic double bonds and their oxidation by the fixative (Criegee *et al.*, 1942; Porter and Kallman, 1953; Bahr, 1954; Wigglesworth, 1957). This reaction at lipidic double bonds may not be the exclusive reaction of OsO_4 with cell constituents since a number of protein side-chains have been demonstrated to interact with the fixative *in vitro* (Porter and Kallman, 1953; Bahr, 1954, 1957; Wigglesworth, 1964).

OsO_4-fixed fibroblasts showed a pale blue fluorescence only when air-dried on crystalline beds of BP (Figs. 121 and 122). The fluorescence of these preparations was totally quenched after about 4 minutes of continuous exposure to u-v light. OsO_4-fixed fibroblasts not treated with BP also have a pale blue fluorescence, but this fluorescence was quenched so rapidly photomicrographs were unobtainable. In comparison, living fibroblasts showed no autofluorescence, and unfixed, air-dried fibroblasts fluoresced with a greenish-yellow color which did not quench (Fig. 124).

OsO_4-fixed fibroblasts, when mounted in an aqueous medium on crystalline beds of BP, showed no demonstrable uptake of BP anywhere within the cell. Crystals of BP displayed a greenish fluorescence, instead of the usual white or blue color. In preparations of OsO_4-fixed cells washed with running tap water for one to two days, the crystalline BP added subsequent to fixation showed the conventional blue fluorescence but no BP was demonstrable within the fixed fibroblasts. OsO_4-fixed cells treated for two to three hours with 3% hydrogen peroxide also showed no demonstrable accumulation of BP.

4% Glutaraldehyde. The 4% glutaraldehyde fixation of L-929 produced relatively little alteration in the live-cell appearance, except in the nuclear region where nucleoli were slightly refractile.

As in nuclei fixed with HCHO solutions, there was a loss in resolvability of chromatin structure (Fig. 117). Cytoplasmic vacuoles were found occasionally (Fig. 117).

In the glutaraldehyde fixed cells, the carcinogen, BP, was

concentrated in the Golgi granules at relatively higher levels than in the general cytoplasmic matrix (Figs. 117, 118). Neither mitochondria nor cytoplasmic vacuoles showed any apparent uptake of BP (Figs. 117, 118). No demonstrable uptake of BP was discernible in any nuclear structure (Figs. 117, 118). With the exception of some cytoplasmic vacuoles, formed as a result of fixation, the general pattern of intracellular BP resembled that in living cells (Figs. 1, 11).

Uptake of the Non-carcinogenic Hydrocargon, Pyrene, by Cells Fixed by Various Agents

In comparison of the results of exposure of Pyrene to cells preserved with a variety of fixatives, the intracellular pattern of this non-CH differed from that of the carcinogen.

Relatively low over-all affinity for Pyrene was displayed by all fixed cells, as was the case with living cells (viz. Figs. 31 and 34). Fibroblasts fixed in 95% EtOH or dichromate showed no photographically demonstrable accumulation of Pyrene after four minutes exposure time. Cells fixed in mercuric chloride, Zenker's fluid or different HCHO-containing fixatives showed only trace amounts of Pyrene accumulation, (Figs. 60, 62, 101, 103, 108).

Evaluation of the specific local sites of the intracellular distribution of Pyrene was difficult. Recognizable vacuolar structures and unidentifiable granules in the cytoplasm showed no demonstrable Pyrene uptake (Figs. 60, 62, 101, 103, 108). Pyrene was not accumulated in apparent Golgi granules to the same extent as that of BP in cells fixed with HCHO-containing fixatives (Figs. 60, 62) or with

Zenker's fluid (Fig. 101). All nuclear structures, including the nuclear membrane, nucleoli and non-nucleolar karyoplasm, failed to show evidence of Pyrene uptake whether the cells were living (Figs. 33, 36) fixed with HCHO-containing fixatives (Figs. 60 and 62), Zenker's fluid (Fig. 101), or mercuric chloride (Fig. 108).

Effects of Fixing with HCHO Vapors on the Intracellular Distribution of Carcinogenic Hydrocarbon

L-929 fibroblasts were fixed by exposure to an atmosphere of HCHO-vapors, then exposed to BP and observed by integrated phase-contrast and u-v-fluorescence microscopy.

In appearance, vapor-fixed cells were markedly altered in comparison with the live-cell appearance. The cytoplasm of most cells was filled with vacuolar structures. The nucleus showed a loss of resolvable chromatin structure (Fig. 39).

Studied with integrated phase-contrast and u-v-fluorescence microscopy, Golgi granules showed the highest apparent BP uptake, while vacuolar structures showed the lowest CH accumulations relative to that of the cytoplasmic matrix (Figs. 39-40). There was no demonstrable BP uptake in any nuclear structure (Figs. 39, 40). This general pattern of intracellular BP is similar to that observed in living cells (Figs. 1-11).

Effects of Sequential Freezing and Fixing on the Intracellular Distribution of Carcinogenic Hydrocarbon

Of the different freezing methods combined with Lillie's HCHO fixation, the slow freezing method produced badly broken cells, in which study relative to the intracellular distribution of BP was not

done. In cells processed by the rapid freezing procedures and subsequently fixed in Lillie's HCHO, the greatest modification of structure was found in those directly immersed in liquid nitrogen. The cytoplasm of these cells was centrally filled with vacuoles, and a fibrous reticlum was apparent peripherally (Fig. 71). Nucleoli were not evident in the nuclei of these cells, and the general intranuclear region was filled with irregular dark strands (Fig. 71).

Cells processed by rapid freezing directly in isopentane at -196°C or by immersion of coverslip-containing culture vessels in liquid nitrogen displayed less structural alteration. The only major modifications, relative to the live-cell appearance, were the appearances of some cytoplasmic vacuolation in central regions and a loss of resolution of chromatin structure (Figs. 68, 70).

The intracellular distribution of carcinogenic hydrocarbon in cells frozen and then fixed was essentially similar, regardless of the rapid freezing procedure used. The nuclei of all cells processed with all the rapid freezing procedures showed no demonstrable accumulations of BP in any nuclear structure (Fig. 67, 72). Vacuolar structures and mitochonria, if observable, showed no apparent BP uptake, while Golgi granules displayed levels of apparent BP uptake that were greater than those in the general cytoplasmic matrix (Figs. 67, 72). This pattern of intracellular BP resembled that in living cells (Figs. 1-11) with the exception of BP distribution relative to structures produced by action of the freezing and fixation procedure itself, such as the cytoplasmic vacuoles.

Effects of Two Post-Fixation Procedures on the Intracellular Distribution of Carcinogen

Cells treated in sequence with Lugol's iodide-iodate solution and thiosulfate after fixation in $HgCl_2$-containing fixatives (fresh Zenker's fluid, Figs. 77, 80; aged Zenker's fluid, Fig. 86; Zenker's stock, Fig. 93; and $HgCl_2$; Fig. 106) showed the same structural appearance as those in control preparations not treated with Lugol's solution and thiosulfate (Figs. 74, 75, 84, 93, 104). The intracellular patterns of BP before Lugol's and thiosulfate treatment or after the post-fixation treatment were the same for each specific fixative tested (Figs. 73-76, 77-80, 84-85, 88-91, 92-93, 103-104, 105-106).

EtOH dehydration and toluene clearing produced marked alterations in the structural appearance of $CaCl_2$-HCHO-fixed L-929 fibroblasts. In the dehydrated and cleared cells, the cytoplasm was filled with precipitated masses and strands of unidentifiable material and vacuolar structures (Figs. 63, 65). The nucleoli were made highly refractile (Figs. 63, 65).

Major qualitative changes in the pattern of intracellular BP, relative to those in living cells (Figs. 1-11) and to $CaCl_2$-HCHO-fixed cells (Figs. 45-49), were induced by the dehydrating and clearing agents. The over-all levels of general protoplasmic uptake of CH appeared to be very low, as judged from the faint fluorescence intensity. In the cytoplasm of these cells, relatively high BP concentrations were noted in the general central cytoplasmic region, in contrast to the peripheral regions. (Figs. 63, 66). Cytoplasmic vacuolar

structures showed no apparent uptake of BP (Figs. 63, 86). Within the nucleus, nucleoli showed evident BP concentration, although other parts of the nucleus, including the nuclear membrane and the non-nucleolar karyoplasm, showed no apparent affinity for the BP. (Figs. 63, 66).

Discussion

The Intracellular Distribution of BP in Cells Fixed With Single and Multiple-Agent Fixatives and/or Post-Fixation Processing

These studies have demonstrated a pattern of intracellular BP distribution which is common to all cells fixed with most of the multiple-and single-agent fixatives tested.

Cells fixed with fresh or aged Zenker's fluid, the various Zenker components (e.g. dichromate, and $HgCl_2$), the various Zenker's components in various combinations (e.g. acidified dichromate and Zenker's stock solution), the various simple HCHO-containing fixatives, Bouin's fluid and Lewitsky's fluid all showed no demonstrable accumulation of BP in any nuclear structure, including the nuclear membrane, nucleolus, chromatin and other Karyoplasmic regions. The accumulation of BP in the cytoplasmic matrix in all three cytoplasmic regions appeared to vary with the type of fixative used. Relatively low BP uptake was demonstrable in cells fixed with $HgCl_2$ and relatively higher uptake appeared in the matrix of cells fixed with the simple HCHO-containing fixatives. A differential distribution of CH was found in the cytoplasm of cells fixed with all of the agents mentioned

above. Lipid droplets and Golgi granules and zones, if preserved, showed comparatively high apparent BP uptake. Cytoplasmic vacuoles and mitochondria, if preserved, showed little BP accumulation. The general pattern of intracellular BP in cells fixed with these single and multi-agent fixatives was similar to that observed in living cells and in all other living nucleated cell types observed (Richter, 1951, 1952a and b, 1955; Richter and Saini, 1960; Shires, 1962; Jones, 1962; Harvey et al., 1963).

Three single-agent fixatives, HOAc, EtOH and OsO_4, effected apparent alterations in the pattern of hydrocarbon which differed from the pattern of CH in cells fixed with other agents. HOAc did not preserve cell structure well enough for study of the intracellular distribution of BP. OsO_4 either degraded intracellular BP or prevented its entry into the cell, disallowing observance of any intracellular fluorescence. Cells fixed in EtOH and exposed to BP displayed the only altered pattern of intracellular BP, compared with that in other fixed and living cells. The distinct point of departure was the evident accumulation of BP by nucleolar and possible other intranuclear structures.

In general, with the exception of EtOH, fixatives which preserved specific cell structures in recognizable form also preserved the pattern of intracellular BP relative to comparable structures in living cells. Comparison of the effects of Zenker's components, singly or in combination, with the effects of complete Zenker's fluid showed no difference in the intracellular distribution of BP among cells

fixed with any of the agents tested.

Compared with the results of the study of CH within cells fixed with single-agent fixatives, the result of study of the intracellular distribution of the non-CH, Py, in cells fixed with the same fluids differed in the following respects: 1) a very much lower apparent affinity for Py than for BP by cells fixed in comparable fixatives, and 2) the absence of demonstrable concentration of Py in Golgi granules in comparison with BP which was relatively more concentrated in these structures than in any other cytoplasmic structure. These differences are somewhat similar to the differences between the intracellular distribution of BP and Py in living L-929 fibroblasts. However, there, as here, both hydrocarbons showed no demonstrable affinity for any nuclear structure.

Other factors in fixation, such as 1) length of fixation time; 2) different physical conditions of fixation, as for example, fixation in vapors or freezing prior to fixation; 3) the sequence of exposure of CH and fixation of cells; and 4) the pHs of comparable fixatives, were not associated with any apparent alteration in the pattern of intracellular BP.

The influence of post-fixation procedures on the intracellular distribution of BP in fixed cells was marked in the case of dehydration and clearing and un-noticeable in the case of the removal of mercury from cells fixed with $HgCl_2$-containing fixatives. Clearing and dehydration of cells fixed with $CaCl_2$-HCHO resulted in a pattern of intracellular BP which included demonstrable uptake of BP by

nucleolar structures. In the study of the post-fixation procedure with Lugol's iodide-iodate solution and thiosulfate, the intracellular patterns of BP were similar in cells fixed in Zenker's fluid, Zenker's stock or in $HgCl_2$, whether this procedure was carried out or not. These results show 1) that mercury in fixatives does not interfere with BP within the cell and 2) dehydration with EtOH and clearing with toluene produce a pattern of intracellular BP which resembles that in EtOH-fixed cells.

Consideration of the Chemistry of Simple Fixing Agents or the Patterns of Intracellular CH

A major observation of this study of the influence of fixation on the intracellular distribution of CH is the alteration of the pattern of BP by EtOH, which was unique among all the fixatives tested. Contrasting the effects of EtOH with other fixing agents in regard to their known chemical interactions with various cell constituents must be limited because of the unavailability of relevant data. In considering some general effects on major groups of biochemical substances, two points can be made a) concerning the intracellular protein and nucleoprotein and b) concerning loss of intracellular lipid.

Very little correlation between general effects on protein and nucleoprotein of fixing agents and the patterns of intracellular BP exists. Of three protein and nucleoprotein precipitators studied (EtOH, $HgCl_2$, chromic acid- as acidified dichromate, Casselman, 1955) and four non-precipitators (HCHO, OsO_4, dichromate, HOAc), HOAc badly damages the L-929 fibroblasts, and OsO_4 apparently interferes with the

uptake or the fluorescence of CH. The precipitating agents, $HgCl_2$ and chromic acid, and the non-precipitators, HCHO and dichromate, preserved the protoplasmic structure of the L-fibroblasts, and the intracellular distribution of CH was essentially similar to that in living cells. EtOH, in contrast to these other precipitators, so altered the cells that BP was accumulated in the nucleoli. Thus the structural state of protein within the cell does not appear to determine the pattern of the intracellular distribution of BP.

Although there are no available data on the comparative total lipid loss in cells fixed with the various agents considered here, it is certain there may be some lipid loss after fixation with any of the agents (Baker, 1960). Chayen et al. (1959) have suggested that loss of lipid from specific structures alters the structures' affinity for BP. Their study involved loss of lipid from cells fixed with $CaCl_2$-HCHO, a fixative reputed to produce little lipid loss (Baker, 1946, 1958). Yet, while other forms of HCHO-fixatives are known to result in loss of significant amounts of lipid (Hamer, 1924; Weil, 1929; Brante, 1949; Wolman and Greco, 1952; Karnovsky and Deane, 1954; Clayton, 1959; Chayen et al., 1960) the patterns of intracellular distribution of CH were essentially the same for all the HCHO-containing fixatives studied, and these patterns resembled those in living cells. The same patterns of intracellular BP were found after fixation with other agents which probably produce lipid loss, such as $HgCl_2$ (Baker, 1960; Baker and Luke, 1962) and after fixation with agents expected to preserve lipid, such as dichromate or chromic acid (Elftman, 1954; Casselman, 1955; Baker, 1960). Probably the best lipid

fixative, OsO_4 (Criegee et al., 1942; Porter and Kallman, 1953; Bahr, 1954; Wigglesworth, 1964), produced cells which showed no apparent BP uptake. Thus, it appears that the simple loss of lipid from fixed cells cannot be used as an explanation for observed alterations in patterns of intracellular BP.

A characteristic which sets EtOH apart from the other simple fixing agents studied is its status as a lipid solvent. If, according to Berg's hypothesis (1951/1952), BP specifically demonstrates sites of intracellular lipid, it could be speculated that EtOH might cause a redistribution of lipid within the cell which would be manifested as an altered pattern of intracellular BP. The question of Berg's hypothesis is part of the concern of the next Chapter.

CHAPTER VI

STUDIES ON THE INTRANUCLEAR DISTRIBUTION OF BP IN ISOLATED LIVER NUCLEI: INFLUENCE OF DIFFERENT MEDIA AND BUFFERS

Introduction

Studies on the intranuclear distribution of BP in isolated nuclei of rat liver have been directed toward two general experimental objectives: a) to assess the effects of different agents conventionally used in nuclear isolation procedures on the intranuclear distribution of BP, and b) to evaluate the influence of a variety of conventional buffer solutions used in various analytic procedures on the incorporation of BP.

The study of the different isolation media's effects on the pattern of nuclear uptake of BP was a comparative one, carried out on nuclei isolated by procedures utilizing the various specific media. These procedures fall into two different categories: a) those using different concentrations of sucrose and b) those using citric acid solutions of different concentrations. This study represents an extension of one carried out by Nordén (1957a).

Study of buffer effects on the intranuclear pattern of BP was based on nuclei which were isolated by a simple sucrose isolation procedure. Nuclei were suspended in various buffers at different pHs and studied comparatively. The types of buffers and pHs selected for testing was suggested a) by their inclusion in CH distribution studies carried out by other workers, e.g. phosphate and acetate buffers (Price et al., 1948, 1949, 1950; Miller, 1951; Weist and Heidelberger, 1953a, b and c; Weisberger, 1953) or b) by their use in incubation media for enzymatic removal of nucleic acid from nuclei, e.g. McIlvaine's buffer and acetate buffer (Stein and Gerade, 1950; Korsin, 1951; Engebring and Laskowski, 1953; Stern and Mirsky, 1953; Daoust and Clermont, 1955; Allfrey et al., 1957; Naora et al., 1961; Nass and Nass, 1963).

Materials and Methods

General Procedures in Preparation for Nuclear Fractionation

Rat liver nuclei were isolated as subcellular fractions. In preparation of these fractions, livers were obtained from adult King-Holtzman rats of both sexes fed ad libitum. They were dispatched by rapid dislocation of the cervical vertebrae. Opening of the abdominal cavity by a midline incision, retraction of the wall and breaching the diaphragm to produce a penumothorax was done with care to prevent contamination of the abdominal viscera with hair. Excised liver fragments were placed in chilled petri dishes containing chilled media and weighted. Each gram of liver was placed in a 10 cc. volume of medium kept cold on ice, and poured into a Dounce glass homogenizer

(after Dounce, 1955) with a moderately tight-fitting ball-type pestle. The homogenizer was fabricated by Mr. Gene May. Approximately 10-15 up and down strokes of the homogenizer were necessary in all cases to acquire a homogenate free of gross tissue fragments. Homogenization was carried out under ice water or in a cold room (2-3^{o}C). The homogenate was strained through 4-6 layers of sterile surgical gauze and rinse prior to filtration with cold media. This step was also carried out in the cold room. After collection, the filtrate was submitted to the various centrifugation procedures of the different isolation methods employed.

The media employed for homogenization were dictated by the isolation procedure being used, but included, 0.25 sucrose, 0.88M sucrose, 2.2M sucrose, 1% citric acid, 1M citric acid.

An alternate homogenization procedure was used for two different methods employing weak citric acid solutions, adjusted to pH 3.8 or pH 6.0. Excised liver fragments were frozen in a refrigerator freezing compartment (approximately -15^{o}C) immediately after removal. The frozen liver fragments were added to carefully adjusted quantities of triple distilled water and weak citric acid solutions in the vessel of a VirTis "23" homogenizer. The machine was run at slow speeds to prevent frothing, and the entire procedure was carried out in the cold room. As homogenization proceeded, the pH, according to the two methods, had to be checked and maintained.

It was found to be extremely important in the preparation of nuclei, using any of the various methods, to properly clean glassware

which came in contact with the nuclear fractions. All glass was washed in Microsolve (Microbiological Associates, Inc.) and after copious rinsing in distilled water, it was de-ionized by rinsing in concentrated nitric acid. Continued copious rinsing in distilled water was followed by several rinses in triple distilled water (Cutter). Just prior to use, all glassware was chilled and rinsed with the medium appropriate to the ensuing experiment.

Specific Nuclear Fractionation Procedures

Isolation in 0.25M sucrose with or without calcium chloride. The procedure involving use of 0.25M sucrose was a modification of the method of Hogeboom, Schneider and Striebeck (1952) and Allfrey et al., (1957). Homogenization was done in a solution of 0.25M sucrose with 0.0018M $CaCl_2$, made up in triple distilled water (Cutter). 10 ml. of medium was used per gm. of tissue. The homogenate was poured onto 4-6 layers of sterile surgical gauze, previously rinsed in sucrose, and allowed to filter slowly. The filtrate was poured into 10 ml. centrifuge tubes and spun at approximately 150 x G for 10 minutes in an International Clinical Centrifuge, Model CL, to sediment most of the unbroken cells. The supernatant was spun at approximately 380 x G for 10 minutes, and its sediment represented the nuclear fraction. Resuspension of nuclear pellets in 0.25M sucrose-$CaCl_2$ solutions and resedimentation was carried out several times to remove as much non-nuclear contamination as possible from the pellet.

In a second experimental procedure using 0.25M sucrose, all details in the isolation of liver nuclei were identical with the above

method except that $CaCl_2$ was omitted from all sucrose solutions employed.

Isolation in 0.88M sucrose with or without calcium chloride. The procedure of Schneider and Peterman (1950) was followed in the isolation of nuclei in 0.88M sucrose solutions, made with triple distilled water (Cutter) and 0.0018M $CaCl_2$. After filtration of the homogenate through sterile surgical gauze, sedimentation of unbroken cells was obtained in the clinical centrifuge at 250 x G for 10 minutes. The supernatant was transferred to lusteroid 10-cc. centrifuge tubes and spun for 10 minutes at 650 x G in an International Refrigerated Centrifuge, Model HR-1, with rotor #260, to obtain a nuclear pellet. Several resuspensions of the pellets in 0.88M sucrose with 0.0018M $CaCl_2$ and resedimentations were carried out to reduce contamination with cytoplasmic constituents as much as possible.

An alternate procedure, after Schneider and Palade (1948), omitted the $CaCl_2$ throughout the entire isolation.

Isolation in 2.2M sucrose solutions. The procedure of Chauveau et al., (1956) was followed. Homogenization in 2.2M sucrose, made up in triple distilled water without $CaCl_2$ was succeeded by centrifugation of the homogenate at 40,000 x G for 60 minutes in an International Refrigerated Centrifuge , head #260.. Although Chauveau et al.,(1956) reported satisfactory isolation of an uncontaminated nuclear fraction after a single centrifuge run, in this study, a second run was carried out because of heavy mitochondrial contamination in this first pellet.

Isolation in 1% and 1.0M citric acid solutions. The procedures are both variations of those described by Dounce (1955), and Calcutt and Payne (1953, 1954), and involved homogenization in citric acid (CA) concentrations of either 1% (.45M) or 1.0M in triple distilled water (Cutter). According to Calcutt and Payne (1953, 1954), homogenates were spun at 1500 x G in an International Refrigerated Centrifuge for 15 minutes, the sediment representing the nuclear fraction. A better method was spinning at 550 x G for 20 minutes in a clinical centrifuge in a cold room. The nuclear pellets of this latter procedure were less contaminated than the former. Nuclear pellets isolated in either 1% or 1M CA were usually washed in appropriate CA solutions only once.

Isolation in citric acid at pH 3.8 and at pH 6.0. Dounce's procedure (1943) for isolation of liver nuclei in a weak solution of citric acid at pH 3.8 was carefully followed in detail. Briefly, the procedure involved freezing fresh rat liver (in a refrigerator freezer) homogenization with a VirTis "23" homogenizer in triple distilled water to which small quantities of 1.0M citric acids were added to maintain a pH of 3.8. The pH was checked and adjusted throughout the homogenization run (about 15 minutes). Centrifugation was carried out at 380 x G for 15 minutes in a clinical centrifuge in the cold room. The procedure for isolation of nuclei in citric acid at pH 6.0 (Dounce, 1950) was essentially similar to the pH 3.8 procedure, except that 0.1M citric acid was used to adjust the pH of the homogenate to pH 6.0.

Nuclear Suspension Media Tested

Once the nuclear pellets had been prepared, three courses of action were followed. 1) Nuclei were isolated and observed in 0.25M sucrose, with/without $CaCl_2$; in 0.88M sucrose, with/without $CaCl_2$; in 2.2M sucrose; in 1.0M or 0.1M citric acid; or in citric acid at pH 3.8 or 6.0. 2) Nuclear pellets prepared by the 0.25M-$CaCl_2$ procedure were suspended in each of the following buffers:

a) 0.2M acetate buffer at pH 4.9 or pH 6.0,

b) McIlvaine's buffer (0.2M sodium dibasic phosphate and 0.1M citric acid) at pH 3.8, pH 4.8 or pH 6.0,

c) 0.1M sodium phosphate buffer (Na-phosphate) at pH 5.5 or pH 7.4,

d) 0.1M sodium phosphate buffer (Na-phosphate) at pH 7.4.

Nuclei in buffers were washed with the individual buffer three times before observation. This was accomplished by repeated suspension and centrifugation of nuclear suspensions at approximately 370 x G for 10 minutes. Nuclei in the lipid solvent were similarly washed in solvent three times, but following this, they were returned to 0.25M sucrose for observation.

Correlative Information Concerning the Condition of Nuclei

In order to provide correlative information concerning the condition of isolated nuclei under examination relative to the patterns of BP uptake, three routine procedures were carried out on the final pellets.

1. Liver nuclei from all of the different isolation procedures were tested for the presence of DNA by the Feulgen reaction (Feulgen and Rossenbeck, 1924; Bauer, 1943). The procedure used was a modification of that followed by Hutchison et al. (1958). Smears of the various nuclear pellets were air-dried, fixed in Lillie's buffered-HCHO, hydrolyzed in 1N HCl for 4 minutes and treated with decolorized Schiff's reagent (Coleman, 1938). No bisulfite rinses were used.

2. Because nuclear fractions suspended in various buffer solutions were found to elevate the pH, pH measurements of all nuclear fractions suspended in the various buffer solutions under consideration were made. Nuclear pellets washed in buffer solutions as cold suspensions (approximately $2^{o}C$) were measured with a Beckman 9600 Zeromatic pH Meter fitted with a Beckman Thermo-compensator.

3. The ability of nuclear fractions to form gels on addition of an equal volume of 1N NaCl indicates the state of polymerization of nucleoprotein within the nuclei (Dounce, 1955; Dounce et al., 1957; Monty and Dounce, 1958; Dounce and Sarkar, 1960; Roodyn, 1963). Strong gels, which show little tendency to flow, have been considered characteristic of unaltered nucleoprotein (Mirsky and Pollister, 1946; Dounce et al., 1957; Monty and Dounce, 1958; Dounce and Sarkar, 1960), while weaker gels show varying abilities to flow depending on the degree of nucleoprotein degradation. As used in this study, qualitative determinations of the ability to form gels were made with 1M NaCl, adjusted to a pH of 7.0 with 0.1N NaOH, added directly to nuclear pellets in the centrifuge tube, after removal of portions for

other experimental procedures. Strong gels would not flow down a 10cc. tube when it was inverted, while weaker gels would.

Observations

Patterns of BP After Various Nuclear Isolation Procedures After Sucrose Isolation

The appearance of all nuclear pellets isolated in the three sucrose procedures were pinkish in color and did not stick to the wall of the centrifuge tubes. Pellets isolated with 0.25M and 0.88M sucrose without $CaCl_2$ did not disperse well and remained as agglutinated clumps of material. Pellets isolated by these two procedures with $CaCl_2$ dispersed well and were relatively less contaminated with cytoplasmic debris. All sucrose-isolated nuclear pellets were contaminated with some red blood cells, various kinds of other nucleated blood cells, partly broken liver cells and general nondescript cytoplasmic debris. Isolation of liver nuclei in 2.2M sucrose produced the least contaminated pellets.

Phase-contrast microscopic examination of the nuclear pellets isolated in the sucrose solutions showed many roughly spherical nuclei containing one to several nucleoli, a dark nuclear rim with occasional cytoplasmic remnants and a hyaline to slightly differentiated nonnucleolar karyoplasm, as specific features after isolation in 0.25M sucrose-$CaCl_2$ as shown in Fig. 250, 252 (Wilbur and Anderson, 1951; Roodyn, 1963), after 0.88M sucrose-$CaCl_2$ as shown in Fig. 258 (Schneider and Peterman, 1950; Roodyn, 1963), and after 2.2M sucrose as shown

in Fig. 262 (Chauveau et al., 1956; Maggio et al., 1963). Chromatin, having structural appearance like that in whole liver cells, was never found in nuclei isolated by these procedures as, also, reported by other workers (Schneider and Peterman, 1950; Wilbur and Anderson, 1951; Chauveau et al., 1956; Roodyn, 1963; Maggio et al., 1963).

Agglutinated nuclei were found in preparations of 0.25M and 0.88M sucrose free of $CaCl_2$. Nuclei in both of these preparations were clear hyaline spheres, surrounded by a dark nuclear rim and not containing any visible nucleoli (Figs. 254, 260). These observations on nuclei isolated at 0.25M and 0.88M sucrose without $CaCl_2$ agree with those reported by Hogeboom et al. (1948), Wilbur and Anderson (1951), Schneider and Peterman (1953), Philpott and Stanier (1956) and Roodyn (1963).

All nuclei isolated by the different sucrose procedures, with or without $CaCl_2$, were stained with the Feulgen reaction and were capable of forming gels. In general, gel formation was weaker in pellets isolated without $CaCl_2$.

Liver nuclei isolated by all of the sucrose procedures studied, with or without $CaCl_2$, displayed apparently identical patterns of BP distribution. Nuclei isolated in 0.25M sucrose with $CaCl_2$ (Figs. 251, 253) or without $CaCl_2$ (Figs. 255), in 0.88M sucrose with $CaCl_2$ (Fig. 259) or without $CaCl_2$ (Fig. 261) and in 2.2M sucrose (Fig. 270) all showed relatively high BP accumulations in the nuclear rims but no demonstrable BP uptake by any structure within the confines of the rim. In the case of 0.25M and 0.88M sucrose without $CaCl_2$, the

intranuclear distribution of BP is as reported by Norden (1957) and is identical to the distribution patterns in the nuclei of intact living liver cells (Figs. 232, 234, 236, 238). In nuclear fractions, the nuclear rim is a feature which represents the nuclear membrane and associated cytoplasmic remnants which cannot ordinarily be removed (Maggio et al., 1963).

A very light blue autofluorescence was observed in nuclear fractions unexposed to BP. It occurred in preparations from all sucrose isolation procedures. The autofluorescence was limited to masses of contamination debris and was never localized in nuclei.

After Citric Acid Isolation. The pellets of liver nuclei isolated in citric acid solutions were variable in appearance. 1M and 1% citric acid procedures produced pellets which were rusty and which turned white on recentrifugation. Isolation at pH 3.8 and 6.0 produced light pink pellets. The nuclear pellets following all citric acid isolations formed turbid, well-dispersed suspensions. Contamination by cytoplasmic debris and whole cells was virtually absent in all citric acid procedures, but some was noticeable in isolation at pH 3.8 and 6.0.

Liver nuclei isolated in the strong citric acid solutions (1.0M and 0.1M) showed distorted shapes, evidence of nuclear shrinkage and pronounced precipitation of strands and clumps of material in the general karyoplasmic region (Figs. 270, 272, 274) (Dounce, 1950, 1955; Zajdela and Morin, 1952; Roodyn, 1963). Nucleoli were indistinguishable in nuclei isolated in 1% citric acid (Figs. 270, 272), but were

discernible in nuclei isolated in 1.0M citric acid (Figs. 274). Nuclei isolated by citric acid showed the presence of DNA by the Feulgen reaction and formed very weak, if any, gel.

Nuclei isolated by weak citric acid pH 3.8 and 6.0 procedures (Dounce, 1950) failed to show the distortion in shape produced by the stronger citric acid solutions (Figs. 264, 266, 268). The general karyoplasmic region of nuclei isolated by both of these procedures was differentiated into strands and granules and generally distinct nucleolar structures (Figs. 264, 266, 268), as reported by previous observers (Dounce, 1943a and b, 1950, 1955; Zajdela and Morin, 1952). The nuclear rims showed occasional tags of cytoplasmic debris (Figs. 264) which were never present on nuclei isolated by the strong citric acid solutions and formed weak gels when treated with neutral saline.

U-V-fluorescence microscopy showed that nuclei isolated by either of the strong citric acid procedures (1% and 1.0M) displayed demonstrable BP uptake in nucleoli and other intranuclear material (Figs. 270, 274). Some of the nuclei isolated in 1% citric acid, however, showed only a diffuse, unlocalized intranuclear uptake of BP (Fig. 272). Relatively high concentrations of BP occurred in the nuclear rims of nuclei isolated in either 1% citric acid (Figs. 270, 272) or in 1.0M citric acid (Figs. 274).

Liver nuclei isolated in the weak citric acid procedures showed patterns of BP uptake which were dissimilar both to those occurring in sucrose-isolated nuclei and in nuclei of whole, living

liver cells (Figs. 2, 3, 5, 9, 11, 251, 253, 255, 259, 261, 263). Perceptible accumulations of CH occurred in nucleoli and other intranuclear masses (Figs. 265, 267, 269). Relatively high accumulations of BP occurred in the nuclear rims (Figs. 265, 267, 269).

Citric acid-isolated nuclei uniformly showed no autofluorescence.

Influence of Buffer Solutions on Isolated Liver Nuclei

Sodium and Potassium Phosphate Buffers. Nuclei suspended in Na-phosphate buffer at pH 5.5 (fraction pH 6.4) showed structural variations which mainly involved the non-nucleolar karyoplasm. The latter was coagulated in some nuclei (Figs. 294, 296, 298) and hyaline in others (Fig. 304). In nuclei with coagulated karyoplasm, the nuclear rim appeared wrinkled (Figs. 294, 296, 298). The nucleoli were compact and phase dark and were discernible in all nuclei suspended in pH 5.5 Na-phosphate (Figs. 294, 296, 298, 304).

Nuclei suspended in pH 7.4 Na-phosphate buffer (fraction pH 7.2) differed greatly in appearance from those suspended in Na-phosphate buffer at pH 5.5 (Figs. 294, 296, 298, 304) and also from the nuclei isolated in 0.25M sucrose with $CaCl_2$ (Figs. 250, 252). In pH 7.4 Na-phosphate buffer, the nuclei were clear hyaline spheres without discernible nucleoli or other internal structural differentiation, but the nuclear rim was discrete (Figs. 300, 302).

The nuclei suspended in K-phosphate buffer at pH 7.4 (fraction pH 7.1) closely resembled nuclei isolated in 0.25M sucrose-$CaCl_2$

(Figs. 250, 252) and some of those in Na-phosphate suspensions (Figs. 304).

The formation of blebs from the rims of nuclei suspended in all the phosphate buffers studied was of frequent occurrence (Figs. 294, 296, 302, 304, 306). This observation appears to confirm a similar finding by Anderson (1953a).

Nuclei suspended in the Na- or K-phosphate buffers showed positive Feulgen reactions (for DNA) irrespective of the specfic pH in all of the phosphate buffers considered (Table I). Strong nucleoprotein gels were obtained from nuclei suspended in Na- and K-phosphate buffers at pH 7.4, while weak, watery gels were obtained from nuclei suspended in Na-phosphate at pH 5.5 (Table I).

Nuclei suspended in all of the phosphate buffers and at all pH levels studied showed essentially similar patterns of accumulation of the CH, BP. All showed relatively high concentrations of the CH in the nuclear rims (Figs. 295, 297, 299, 301, 303, 305, 307, 309). There was no demonstrable accumulation of BP within the limits of the nuclear rim in nuclei suspended in Na-phosphate at pH 5.5 and in K-phosphate at 7.4 (Figs. 295, 297, 299, 306, 307, 309). In Na-phosphate at pH 7.4, some nuclei showed some possible BP accumulation in the general karyoplasm (Fig. 301), but others (Figs. 303) did not despite their similar structural appearance in phase-contrast microscopy (Figs. 300, 302). With these occasional exceptions the patterns of BP accumulated by nuclei suspended in the various phosphate buffers resembled those by nuclei isolated in 0.25M

TABLE I

STUDIES OF SUCROSE NUCLEI RESUSPENDED IN BUFFER SOLUTIONS

	Buffer	Tissue pH*	Feulgen	Gel	BP Uptake
1.	McIlvaine's pH 6.0	7.1	yes	none	rim only
2.	McIlvaine's pH 4.8	5.4	yes	none	rim only
3.	McIlvaine's pH 3.8	4.5	yes	none	rim only
4.	Acetate pH 6.0	6.8	yes	none	throughout
5.	Acetate pH 4.9	6.0	yes	none	throughout
6.	Na Phosphate pH 7.4	7.2	yes	strong	rim only
7.	Na Phosphate pH 5.5	6.4	yes	weak	rim only
8.	K Phosphate pH 7.4	7.1	yes	strong	rim only

* Represents an average of three determinations on at least three different preparations.

sucrose-$CaCl_2$ (Figs. 251, 253).

Blebs associated with the rims of isolated nuclei suspended in phosphate buffers showed characteristic accumulations of BP in their walls but no demonstrable BP accumulation by the intra-bleb material which is of uncertain nature (Figs. 295, 297, 299, 303, 305, 307) (Anderson, 1953a).

Acetate buffers. Nuclei suspended in 0.1M acetate buffers at either pH 4.9 (fraction pH 6.0) or pH 6.0 (fraction pH 6.8) were of similar appearance, but which differed from that of nuclei isolated in 0.25M sucrose-$CaCl_2$ (Figs. 250, 252). The non-nucleolar karyoplasm was filled with finely agglutinated materials, associated with one to several clear spaces per nucleus (Figs. 285, 287, 289, 291, 293). The margins of all nuclei were refractile and the nuclear rims tended to be wrinkled (Figs. 285, 287, 289, 291, 293). No nuclear blebs were found in nuclei suspended in acetate buffers.

Nuclei suspended in acetate buffers at pH 4.9 and pH 6.0 gave positive Feulgen reactions (Table I). Neutral saline did not elicit any gel-formation by nuclei in acetate buffer at either pH 4.9 or 6.0. (Table I).

On exposure to BP, the suggestion of an intranuclear accumulation of the fluorescent CH was noted which appeared to be spread evenly throughout the karyoplasmic region of nuclei suspended in acetate buffer at pH 4.9 and 6.0 (Figs. 286, 299, 290, 292).

The intranuclear uptake of BP appeared to be more pronounced at the lower pH (Figs. 288, 290, 292 vs. Fig. 286). Accumulation of BP in the nuclear internum was similar to that observed in some nuclei

in preparations suspended in Na-phosphate buffer at pH 7.4 (Figs. 301) and as was the case there, no differential accumulation by the different structures within the confines of the nuclear rim was detected (Fig. 286, 288, 290, 292). The nuclear rims of all nuclei suspended in the different acetate buffers showed relatively higher accumulations of BP than the interior material (Figs. 286, 288, 290, 292). The suggestion of BP uptake by the material in the nuclear interior was an alteration of the pattern observed in nuclei in 0.25M sucrose-$CaCl_2$ (Figs. 251, 253).

McIlvaine's buffer. Liver nuclei suspended in McIlvaine's buffer at pH 3.8 or pH 4.8 were observed to have one to several dark compact nucleoli amid large clumps of non-nucleolar karyoplasm (Figs. 276, 278, 279). The non-nuclear karyoplasmic material in nuclei suspended in McIlvaine's buffer at pH 6.0 (fraction pH 7.1) was nearly homogeneous (Figs. 281, 283). At all pH values the margins of nuclei were somewhat refractile, but discernible nuclear rims and tags of cytoplasm were seen (Figs. 276, 279, 281). Some slight distortion of nuclear shape and wrinkling of the nuclear surface was observed in nuclei suspended in McIlvaine's buffer at the two more acid pHs (Figs. 276, 278, 279). No blebbing from the surface of McIlvaine's buffer-suspended nuclei at the three pHs studied was observed.

Nuclei suspended in McIlvaine's buffer (pHs 3.8, 4.9 and 6.0) gave positive Feulgen reactions (Table I), but none formed nucleoprotein gels when exposed to neutral saline (Table I).

The intranuclear distribution of BP in nuclei suspended in

McIlvaine's buffer at the three pHs used was similar to that of nuclei isolated in 0.25M sucrose-$CaCl_2$ (Figs. 251, 253). There was no demonstrable uptake of BP by any structure within the confines of the nuclear rim, including nucleoli, and non-nucleolar karyoplasm (Figs. 277, 280, 282, 284). Demonstrable BP localization occurred in the nuclear rims and cytoplasmic tags of all nuclei suspended in McIlvaine's buffer solutions at the pHs of 3.8, 4.9 and 6.0 (Figs. 277, 280, 282, 284).

Discussion

Comparative examination of the distribution of BP in liver nuclei isolated and observed in two groups of media (citric acid and sucrose) showed patterns of BP uptake which were highly characteristic of each group. In general, nuclei isolated in all of the different sucrose media showed no demonstrable BP uptake by any structure within the confines of the nuclear rim, while nuclei isolated in the various citric acid solutions showed BP uptake by various karyoplasmic structures. The BP patterns observed in sucrose nuclei resembled that in nuclei in intact living hepatic parenchymal cells.

Within the citric acid groups some variations in the pattern of BP uptake were noted between nuclei isolated in the two strong citric acid solutions tested and the two weak citric acid solutions. In strong citric acid the karyoplasm was markedly coagulated in discrete masses which localized BP, whereas in the two weaker citric acid solutions, karyoplasmic material was not so completely confined to distinct masses and BP was consequently more diffusely situated.

Within the sucrose group of media, nuclei showed the same BP uptake patterns despite specific modifications in the sucrose isolation media, which are known to have specific effects on nuclear composition, structure and function.

1) The inclusion of specific concentrations of $CaCl_2$ in media is known to be important to holding cytoplasmic contamination of nuclear fractions to a minimum (Schneider and Peterman, 1950; Wilbur and Anderson, 1951; Hogeboom et al., 1952; Philpott and Stanier, 1956) and to the maintenance of nucleoprotein and chromosomes by preventing their hydration and consequent gel formation (Anderson and Wilbur, 1952; Bernstein and Mazia, 1953; Schneider, 1955; Monty and Dounce, 1955; Philpott and Stanier, 1956, 1957; Anderson and Norris, 1960). The observation that lack of $CaCl_2$ in various sucrose suspension media did not alter the nuclear pattern of BP uptake, although it did alter the morphology of the nucleus, suggests that $CaCl_2$ is not a controlling factor in distribution of intranuclear BP.

2) Sucrose concentration: Through its tonicity sucrose concentrations influence both a) nuclear ultrastructure and b) nuclear physiology. a) Preservation of the ultrastructural characteristics of nuclei in intact liver cells apparently has not been achieved after isolation in isotonic (0.25M) or slightly hypertonic (0.88M) sucrose media (Allfrey and Mirsky, 1955; Davidson and Mercer, 1957; Bornig et al., 1960). Those nuclei isolated in more hypertonic sucrose media (0.2M) were similar in appearance to nuclei in intact cells (Chauveau et al., 1956; Hinegardner, 1962; Sporn et al., 1962;

Maggio et al., 1963; Mariano, 1964; Gill, 1965). b) The protein synthesis (Allfrey et al., 1957, 1961, 1964; Allfrey and Mirsky, 1958, 1962; Hopkins et al., 1961; Chipchase et al., 1963) and nucleic acid synthesis (Friedkin and Wood, 1956; Osawa et al., 1957; Allfrey et al., 1957, 1961, 1964), observed in nuclei isolated in 0.25M sucrose-$CaCl_2$, is halted by resuspension in hypertonic sucrose solutions (Allfrey et al., 1957). In this study and others (Schneider and Peterman, 1950; Wilbur and Anderson, 1951; Philpott and Stanier, 1956; Roodyn, 1963), nuclei isolated in sucrose at 0.25M, 0.88M and 2.2M appeared similar at the light microscopic level and, further, as shown here, present identical patterns of nuclear BP accumulation. It would appear that the patterns of BP distribution in sucrose isolated nuclei is not directly referable to sucrose concentration or indirectly to sucrose-induced alterations in nuclear structure and protein and nucleic acid synthesizing capabilities.

As shown here, nuclei isolated in citric acid are characterized by specific nuclear and diffuse karyoplasmic accumulations of BP, patterns totally unlike those in sucrose isolated nuclei. The chemical basis for this difference in BP distribution is not evident beyond the point that it is due somehow to a specific effect(s) of citric acid. The composition of sucrose-isolated nuclei and citric acid-isolated nuclei are undoubtedly different, but how much so is not clearly established. Sucrose isolated nuclei retain small molecules such as mononucleotides (Osawa et al., 1957), divalent cations (Naora et al., 1961), some soluble enzymes (Stern and Mirsky, 1953) and sustained activity of a wide variety of intranuclear enzymes (Hogeboom et al., 1948,

1952; Schneider, 1948; Schneider and Hogeboom, 1950; Palade, 1951; Siebert and Smellie, 1957; Roodyn, 1959, 1963) and apparently lose some protein (Stern and Mirsky, 1953; Kay et al., 1956; Hale, 1956). Citric acid isolated nuclei apparently lose significant amounts of protein, amino acids, RNA and mononucleotides (Dounce, 1950; Allfrey et al., 1952; Kay et al., 1956; Hale, 1956; Naora et al., 1957). The activity of many enzymes is retained in citric acid-isolated nuclei (Dounce, 1943 a and b, 1950, 1955; Emery and Dounce, 1955a and b; Siebert and Smellie, 1957; Roodyn, 1959, 1963), but citric acid is a known inhibitor of many other enzymes, including DNAase (McCarty, 1946; Mirsky and Ris, 1950/1951). It has been suggested too, on histochemical grounds that 2-5% citric acid solutions unmask lipoprotein (Brock et al., 1952; Ackerman, 1952). On the basis of the limited date available on the comparative compositions of sucrose-isolated and citric acid effects extensive nuclear biochemical alterations which include: a) the loss of some components such as RNA and b) the possible unmasking of bound lipid, both elements which have been considered to play significant roles in the general intranuclear distribution of CH.

Collectively considered, the data on the influence of buffers and isolation media on the distribution of BP in isolated nuclei warrant the following suggestions.

1) The intranuclear or karyoplasmic accumulation of BP or lack of it by isolated nuclei suspended in acetate, Na-phosphate and McIlvaine's buffer appears not to be dependent on pH.

2) The intranuclear accumulation of BP or lack of it in isolated nuclei appears not to be dependent on modification of the molecular (polymeric) state of nuclear nucleoprotein (as indicated by the gel formation tests) effected by different buffers at multiple pHs.

3) The intranuclear accumulation of BP or lack of it in isolated nuclei appears not to be dependent on overt physical modifications of karyoplasmic components as reflected by differences in the appearance of the karyoplasmic coagulum.

4) The intranuclear accumulation of BP (as in citric acid-isolated nuclei and in acetate buffered-nuclei) or the lack of it (as in the other isolation media and the other buffers as noted above) appears to be independent of the presence of DNA, as evidenced by the fact that all nuclei, irrespective of the isolation media or buffer used, yielded positive Feulgen Tests for DNA.

5) The intranuclear accumulation of BP or lack of it by isolated nuclei appears to be referable to the presence of anions common to citric acid and acetate buffer solutions.

CHAPTER VII

STUDY OF THE INFLUENCE OF LIPID ON THE INTRACELLULAR DISTRIBUTION OF CARCINOGENIC HYDROCARBON

Introduction

Study of the influence of lipid on the intracellular distribution of CH was provoked by Berg's hypothesis (1951/1952) that BP histochemically demonstrated all intracellular lipid, bound or unbound. Berg's studies (1951/1952) utilized limited lipid extraction procedures. In the present studies, the influence of lipid on CH distribution was evaluated via methods striving toward total lipid extraction of cell test material.

Procedurally, L-929 fibroblasts, exposed and unexposed to BP *in vitro*, are submitted to extraction by the 1:1 (v/v) chloroform ($CHCl_3$) and methanol (MeOH) mixture of Folch-Pi and Lees (1951) or to extraction with warm pyridine (Baker, 1946). $CHCl_3$:MeOH has been found to remove lipid almost *in toto* (Folch-Pi and Lees, 1951; Lovern, 1955; Hanahan, 1960; Entenman, 1961), while warm pyridine is primarily a phospholipid solvent (Baker, 1946; Lovern, 1955). Both solvents have been extensively used in histochemical work (Baker, 1946, 1949; Casselman, 1952; Karnovsky and Deane, 1954; Chayen *et al.*, 1957;

Berenbaum, 1958; Almeida and Pearse, 1958).

The analytic approaches involved were: 1) the extraction of L-fibroblasts after growth on BP beds by either warm pyridine or $CHCl_3$:MeOH, 2) the extraction of cells not grown in media containing BP, and 3) the extraction of isolated liver cell nuclei by $CHCl_3$:MeOH.

In the first approach, L-cells grown on BP *in vitro* were fixed with any of four fixatives and extracted with either warm pyridine or $CHCl_3$:MeOH.

In the second approach, the cultured fibroblasts were fixed as in the first approach and extracted with $CHCl_3$:MeOH. They were then exposed to BP via Berg's caffeine-BP solution (1951/1952).

In the third approach, liver nuclei, freshly isolated in sucrose media, were exposed to BP in aqueous solutions and to Berg's solution, either singly or in combination, after lipid extraction by $CHCl_3$:MeOH.

The experimental approaches used were expected to establish whether the intracellular distribution of BP was related exclusively to protoplasmic lipid (bound or unbound) or to other non-lipid protoplasmic components, e.g. protein (Miller, 1951; Moodie *et al.*, 1954; Woodhouse, 1954, 1955; Carruthers *et al.*, 1957), or to other unknown factors.

Materials and Methods

Removal of the general lipid content of L-cells and isolated liver nuclei was attempted by two different procedures, one involving

use of $CHCl_3$:MeOH and the other using warm pyridine. Extractions of cells or nuclei which had been exposed to CH had to be carried out in separate vessels from the control preparations because of the high solubility of the CH in these solvents. The control cells and nuclei, unexposed to BP, were handled in every way like the experimental lipid-extracted material.

Extraction of L-929 Fibroblasts

The procedure using a 3:1 chloroform to methanol mixture ($CHCl_3$:MeOH) is a modification of that used by Berenbaum for extraction of total lipid (1958). L-cells were fixed approximately one hour in Bouin's fluid, 95% EtOH, a 3:1 (v/v) $CHCl_3$:MeOH solution or Baker's $CaCl_2$-HCHO solution, using the previously described techniques. After thorough washing in running tap water and several changes in distilled water, the cells were immersed in 3:1 $CHCl_3$:MeOH in clean Columbia staining dishes at room temperature. The solvent mixture was changed hourly for five to twelve hours. On completion of the extraction, cells were returned to water and mounted on clean glass slides, sealed with beeswax and observed, or they were returned to water and treated with Berg's caffeine-BP solution (Berg, 1951/1952) for 20 minutes prior to mounting.

Prolonged continuous extraction with $CHCl_3$:MeOH was carried out in a jacketed Soxhlet apparatus, designed by Lhotka (1955). The extraction chamber was covered with aluminum foil to protect against photodegradation of the CH (Woenckhaus et al., 1962; Karatsune et al., 1962).

Fixed L-cells were transferred from $CHCl_3$:MeOH to the extraction chamber which was also filled with $CHCl_3$:MeOH, special care being taken in all these transfers to avoid drying of the preparations. Extraction periods were one and two days, and one and two weeks, with daily renewal of the extracting fluid.

The procedure for removal of lipid with warm pyridine was a modification of that recommended by Baker (1946). This involved Bouin's fluid fixation, followed by one hour in 70% EtOH, 1/2 hour in 50% EtOH and 1/2 hour in running tap water, and then transfer to pyridine at room temperature for 1/2 hour followed by a change to pyridine at 60^0 for approximately 5, 12, 24 and 48 hours. The extracted cells were then rehydrated and observed immediately or treated with Berg's BP solution and then observed.

Extraction of Isolated Liver Nuclei

Liver nuclei, isolated in 0.25M sucrose-$CaCl_2$ by methods previously described, were suspended in $CHCl_3$:MeOH and the extracting solvent renewed three times by sedimentation of nuclear pellets at 350 x G for 10 minutes in the cold room (2^oC), followed by resuspension in fresh solvent. The over-all length of exposure to $CHCl_3$:MeOH was approximately 50 minutes. The extracted nuclear pellets were washed in water several times and wet slide preparations made by procedures previously described.

Observations

Effect of lipid extraction on the apparent BP content of Cells Grown in vitro on the Hydrocarbon

Extraction with $CHCl_3$:MeOH. Cells extracted with $CHCl_3$:MeOH for 5-12 hours showed relatively little alteration in cytomorphologic appearance as compared with unextracted cells. In these 5-12 hour-extracted cells, the residues of Golgi zones, Golgi granules and other unidentifiable granular, vesicular and rod-shaped cytoplasmic structures were clearly observed in HCHO-fixed cells (Figs. 181, 183, 185), EtOH-fixed cells (Figs. 191, 192), $CHCl_3$:MeOH-fixed cells (Figs. 173, 175, 176) and Bouin's-fixed cells (Figs. 167, 168, 169, 170). The nuclei of these cells generally resembled those in unextracted cells (Figs. 167, 168, 169, 170, 173, 175, 176, 181, 191, 192).

Cells fixed in Bouin's fluid, $CHCl_3$:MeOH or EtOH and extracted for 20 hours or more with $CHCl_3$:MeOH displayed highly refractile cytoplasm and nuclei, in which many residual structures appeared obliterated by interference patterns (Figs. 171, 179, 193, 195, 197). There was evidence of marked cell shrinkage in extracted, EtOH-fixed, spindle-shaped L-fibroblasts (Figs. 183, 195, 197) and breaks in the continuity of cytoplasmic processes in extracted Bouin's-fixed cells (Fig. 171). HCHO-fixed cells notably sustained less damage for extended $CHCl_3$:MeOH extraction relative to their cytomorphology at the light microscopic level (Figs. 185, 187).

One feature of $CHCl_3$:MeOH extraction of fixed cells worthy of special mention was the greater extractability of the nuclear membrane

in cells not grown on BP than in those cells exposed to BP. As seen in cells extracted for periods greater than 20-24 hours, the complete or partial absence of a discernible nuclear membrane was most pronounced in cells not grown on BP beds and fixed with $CHCl_3$:MeOH (Figs. 177, 215) or EtOH (Figs. 191, 193, 195, 197), but similar degrees of nuclear membrane extraction also occurred with lesser frequency in cells fixed with Bouin's fluid (Figs. 171). Quantitative data on the protective effect of BP against $CHCl_3$:MeOH extraction of nuclear membranes are shown in Table II. The nuclear membranes in cells grown on BP and extracted for short periods of time (five to twelve hours) infrequently showed breaks in their continuity. Nuclear membranes were generally intact in HCHO-fixed and $CHCl_3$:MeOH extracted cells, grown or not grown on BP, and subjected to a full range of extraction times (Figs. 181, 183, 184, 185, 187).

As summerized in Table III, apparent removal of all intracellular BP, or its fluorescent derivatives, was obtained with $CHCl_3$:MeOH-extraction for 1) five hours, in the cases of cells fixed in Bouin's fluid (Figs. 167, 168) and cells fixed with $CHCl_3$:MeOH (Figs. 175, 176), or 2) for 12 hours, in the cases of cells fixed in EtOH (Figs. 191, 192) and in $CaCl_2$-HCHO (Figs. 183, 184). Both EtOH- and $CaCl_2$-HCHO-fixed cells extracted for only five hours showed a very light fluorescence that was visible on low magnifications (Figs. 181, 182 189, 190). Cells fixed and extracted in $CHCl_3$:MeOH for three to five hours showed an indistinct differential distribution of residual BP in the cytoplasm, but no residual CH in the nucleus, except for distinct localization of the bluish fluorescence in the nucleoli (Figs.

TABLE II

EFFECT OF CH IN CONFERRING RESISTENCE TO EXTRACTION OF NUCLEAR MEMBRANES OF L-FIBROBLASTS WITH LIPID SOLVENTS*

Fixative	Total Number Preparations	Total Nuclei Observed	Intact Nuclei** Observed	% Intact Nuclei
$CHCl_3$:MeOH				
1. Grown on BP	6	954	850	89%
2. Not Grown on BP	4	598	366	61%
EtCH				
1. Grown on BP	5	661	541	82%
2. Not Grown on BP	4	943	207	22%

* All the preparations in this Table were extracted in a Soxhlet apparatus for five to seven days.

** The operational definition of "intact" nuclear membrane was the unbroken continuity of the structure over its entire circumfrence.

173, 174).

Control preparations, not exposed to BP beds in vitro, showed no apparent fluorescence within cells prepared with any of the four fixatives and extractions of five to twelve hours (Table, III) (Figs. 167, 168, 169, 170, 175, 176, 183, 184, 191, 192).

As shown in Table III, L-929 fibroblasts, not exposed to BP and extracted with $CHCl_3$:MeOH for 24 hours or more, showed a light blue intracellular autofluorescence in preparations involving all four fixatives (Figs. 172, 178, 180, 186, 194, 198). The intracellular pattern of autofluorescence was similar in all preparations, except those fixed with $CaCl_2$-HCHO, in which u-v light quenched the fluorescence too rapidly for photography (Figs. 185, 186, 187, 188). In all other preparations, the fluorescence was localized in the general cytoplasmic precipitate (Figs. 172, 179, 180, 194, 198). The only fluorescence associated with the nucleus was apparently situated in the nucleolar residue (Figs. 172, 179, 180, 194, 198).

In summary, extraction periods of five to twelve hours appeared to remove all detectable fluorescence (Table III). Controls for these periods were also non-fluorescent, but if extraction proceeded beyond 12-24 hours, these preparations, which were unexposed to BP, became autofluorescent.

Extraction with warm pyridine. L-929 fibroblasts, extracted by this modification of Baker's procedure (1946) involving extraction with warm pyridine for from 5-24 hours after Bouin's fixation, produced a variably heavy precipitate of cytoplasmic and nuclear material.

TABLE III

EXTRACTION OF BP FROM CELLS WITH CHLOROFORM:METHANOL

Fixative	Fluorescence	Fixative	Fluorescence
Grown on BP		Not grown on BP	
$CaCl_2$-HCHO		$CaCl_2$-HCHO	
5 hrs. extr.	yes	5 hrs. extr.	no
12 hrs. extr.	no	12 hrs. extr.	no
24 hrs. extr.	yes	24 hrs. extr.	yes
EtOH		EtOH	
5 hrs. extr.	yes	5 hrs. extr.	no
12 hrs. extr.	no	12 hrs. extr.	no
24 hrs. extr.	-	24 hrs. extr.	yes
7 days extr.	yes	7 days extr.	yes
Bouin's Fluid		Bouin's Fluid	
5 hrs. extr.	no	5 hrs. extr.	no
12 hrs. extr.	no	12 hrs. extr.	no
24 hrs. extr.	yes	24 hrs. extr.	yes
$CHCl_3$:MeOH		$CHCl_3$:MeOH	
3 hrs. extr.	yes	3 hrs. extr.	-
5 hrs. extr.	no	5 hrs. extr.	no
20 hrs. extr.	-	20 hrs. extr.	yes
7 days extr.	yes	7 days extr.	yes

Thick nuclear rims, dense strands and granules in the cytoplasm, and thick ridges which ran parallel with the axis, sometimes the whole length of the cell, were characteristics of these preparations, whether grown on BP in vitro (Fig. 194) or not (Fig. 201, 202). Portions of the nuclear rim could be found dissolved away, regardless of the history of the cells' exposure to BP or the duration of extraction (Figs. 199, 201, 202, 203). Nucleoli were more or less indistinct because of the heavy intranuclear masses of precipitate (Figs. 199, 201, 202, 203). This characteristic of heavy precipitation of intracellular material seemed an exaggeration of a similar condition found in cells simply fixed with Bouin's fluid. (Fig. 165).

L-fibroblasts, grown on BP and submitted to warm pyridine extraction for five to twelve hours, showed a distinct light blue intracellular fluorescence, differentially situated. The coarsely precipitated cytoplasm showed a generalized retention of apparent BP or its fluorescent derivatives. Slightly higher BP concentrations occurred in structures of the cytoplasm which were unidentifiable, except for the nuclear rim region (Fig. 200). Distinct blue fluorescence was localized in the precipitated intranuclear masses, but not in the intervening regions (Fig. 200).

L-cells not exposed to BP and extracted with warm pyridine for five to twelve hours showed no discernible fluorescence.

L-cells not exposed to BP and extracted for one to two days showed a faint but distinct light blue fluorescence (Fig. 204). This fluorescence, also seen in preparations exposed to BP and extracted for

the same length of time, was not well localized, appearing both in nuclear and cytoplasmic regions with even intensity (Figs. 203, 204). Some granules containing relatively higher intensities of fluorescence could not be related to particular cell structures (Figs. 203, 204).

Thus, cells exposed to BP and extracted with warm pyridine over a full range of extraction times showed intracellular fluorescence. Cells not exposed to BP were non-fluorescent after shorter extraction times but became autofluorescent after 24-48 hours in warm pyridine.

Study of the Intracellular Uptake of BP from Berg's Solution Relative to the Lipid Content of Cells

Unextracted L-cells treated with Berg's solution. Staining fixed cells after Berg (1951/1952) with a caffeine-BP solution resulted in patterns of intracellular fluorescence which varied with the fixative employed. Fixation with Zenker's fluid, Bouin's fluid and EtOH resulted in very faint blue fluorescence and poorly resolvable localization of the CH (Figs. 205, 206, 227, 228, 229, 230). The general intranuclear region of cells fixed with Zenker's fluid appeared, as a rule, to display little affinity for the fluorescent material (Figs. 229, 230). The presumptive nucleoli in EtOH- and Bouin's fluid-fixed cells displayed apparent uptake of the CH (Figs. 205, 206, 227, 228). This localization, in the case of the former fixative, was similar to that observed in EtOH-fixed cells grown on BP beds (Figs. 110, 113). No demonstrable intranuclear uptake of BP from crystalline beds was observed in Bouin's-fixed cells (Figs. 165, 166).

In contrast to the results with these fixatives, study of

$CaCO_3$-HCHO-fixed cells after treatment with Berg's BP-solution revealed an intracellular pattern of BP essentially similar to that in cells exposed to BP beds. All nuclear structures showed no apparent accumulation of the CH (Figs. 52, 53, 54). The general Golgi zone and Golgi granules demonstrated relatively highest uptakes and presumptive mitochondria and other vesicular structures relatively lowest uptakes of BP (Figs. 52, 53, 54).

Effects of dehydration and clearing. Running HCHO-fixed L-cells up through graded alcohols to toluene and back to water, by procedures previously described, resulted in a reduction in the levels of apparent accumulation of BP from Berg's solution (Figs. 225, 226). The general pattern of intracellular BP was similar to that in cells not post-treated, except in regard to the nucleolus, where demonstrable accumulations of BP were regularly observed (Figs. 225, 226). A similar alteration in the intracellular distribution of BP was noted in HCHO-fixed cells, dehydrated, cleared and exposed to BP crystals on slide preparations (Figs. 64, 66).

Effects of lipid extraction. Fluorochromation with Berg's caffeine-BP solution of fixed cells, extracted with $CHCl_3$:MeOH until both those cells grown on BP in vitro and those not grown on BP were non-fluorescent (approx. 5-12 hours), resulted in relatively low but demonstrable intracellular uptake of fluorescent hydrocarbon.

Discernible intranuclear uptake of the fluorochrome by nucleoli and possibly other unidentifiable intranuclear components was found in extracted EtOH-fixed (Figs. 219, 220, 221, 222), Bouin's-fixed (Figs.

207, 208, 209, 210), and $CHCl_3$:MeOH-fixed cells (Figs. 215, 216, 217, 218). Accumulation of fluorescent material by intranuclear structures in extracted $CaCl_2$-HCHO-fixed cells was questionable (Figs. 211, 212) 213, 214). No apparent uptake of BP was displayed by the residual chromosomal material of metaphase plates in EtOH-fixed and $CHCl_3$:MeOH-extracted cells (Figs. 221, 222).

An indication of differential accumulation of the CH from Berg's solution in cytoplasm of fixed and extracted L-cells appeared in u-v-fluorescence photomicrographs (Figs. 207-222). Precise identification of structures in which the relative apparent concentrations of CH varied from those displayed by the general cytoplasm was rendered difficult by the over-all low levels of fluorescence and by the lack of preservation of organelle structure following lipid extraction.

No demonstrable uptake of BP from crystalline beds was observed in cells extracted for five to twelve hours, even after incubation at room temperature for several hours on sealed slide preparations.

Effect of Lipid Extraction on CH Uptake by Liver Nuclear Fractions

Liver nuclei, isolated in 0.25M sucrose-$CaCl_2$ and extracted with $CHCl_3$:MeOH, were highly refractile, irregular in shape and filled with a coarsely precipitated non-nucleolar karyoplasm, containing one to several distinct nucleoli (Figs. 256).

Exposure of extracted nuclei to crystalline beds of BP, to Berg's caffeine-BP solution (1951/1952) or a simultaneous exposure to

both BP crystals and Berg's solution did not result in any demonstrable BP uptake by any structure associated with the isolated liver nuclei (Figs. 257). Some apparent uptake of BP from Berg's solution was noted in large masses of debris in the nuclear fractions studied (Fig. 256). These same masses of debris showed some autofluorescence on control preparations unexposed to BP. No autofluorescence was ever observed in intact nuclei.

Lipid-extracted nuclei yielded positive Feulgen reactions but formed no gel in neutral saline.

Conclusions

The results of this study are brief: 1) $CHCl_3$:MeOH, but not warm pyridine, was shown to extract all fluorescent material from L-929 fibroblasts grown on BP *in vitro* and fixed with any of four fixatives, including $CHCl_3$:MeOH itself. 2) Cells, unexposed to BP *in vitro* and extracted for time periods shorter than those producing autofluorescence, showed a demonstrable uptake of BP from Berg's solution. 3) Extracted isolated liver nuclei failed to show BP uptake, even when exposed to BP crystals and to Berg's solution simultaneously.

The observation that $CHCl_3$:MeOH removes all fluorescent material from within L-cells grown on BP cannot be interpreted unequivocally. It probably demonstrates the absence of strong protein-CH binding involving the residual material of the cell, but the results are uninformative in regard to the binding of CH to protein extracted by the solvent system (Folch-Pi and Lees, 1951; Entenman, 1961). The failure of warm pyridine to remove all fluorescent material could be attributed

to a) incomplete removal of lipid and lipid-associated BP and/or b) failure to remove the same protein which $CHCl_3$:MeOH extracted and to which BP might be bound. These results, thus, do not provide clear-cut evidence for or against the possibility of protein influencing the intracellular distribution of CHs.

The apparent protection of the nuclear membrane against extraction conferred by exposure of the cells might be regarded as evidence for some form of CH interaction with cellular constituents.

The observations that fibroblasts extracted with $CHCl_3$:MeOH accumulated BP from Berg's solution foster at least a suspicion that lipid does not by itself determine the intracellular distribution of CH. Although after 24 hours extraction, the advent of autofluorescence, which is probably due to dehydration (von Jancsó, 1932; Sjöstrand, 1944) prohibited unlimited extraction, it seems probably a great majority of the total lipid would be gone after five to twelve hours extraction. The method of Folch-Pi (1951) utilized contact of fresh tissue with the extracting solvent for only a few minutes in order to obtain complete extraction (viz. Marinetti et al., 1958). In the present study, use of $CHCl_3$:MeOH as a fixative as well as a lipid solvent approximates the Folch procedure.

There are several alternative interpretations for the apparent BP uptake from Berg's solution by lipid-extracted cells. 1) BP might be capable of demonstrating the probably infinitesimal amounts of residual lipid in the extracted cells. 2) Despite Berg's denial (1951/1952), some degree of BP adsorption to lipid-free residual

structures seems possible, since adsorption has been demonstrated for CHs (Brock et al., 1938; Chalmer, 1955). 3) Non-specific, non-vital protein binding of BP to protein might occur in these preparations as suggested by Hadler et al. (1959). Data on which to base a choice of any of these alternatives are at present insufficient.

Because of the very limited available data, interpretation of the comparative observation that lipid-extracted, isolated liver nuclei showed no demonstrable BP uptake while residual nucleolar structures in extracted fibroblast nuclei showed BP uptake must remain incomplete and unsatisfactory.

CHAPTER VIII

STUDIES ON THE INFLUENCE OF NUCLEIC ACIDS ON THE INTRACELLULAR DISTRIBUTION OF CARCINOGENIC HYDROCARBONS

Introduction

The existing literature bearing on the sites of intracellular distribution of CH is in marked disagreement relative to CH accumulation in the nucleus. The position of u-v-fluorescence microscopists that little to no CH accumulates in the karyoplasm of living cells (Graffi and Maas, 1940; Günther, 1941/1942; Waddington and Goodhart, 1949; Richter, 1951, 1952a and b, 1955; Richter and Saini, 1960; Shires, 1962; Jones, 1962; Harvey et al., 1963) is disputed by reports 1) that nucleic acid-bound CH can be isolated directly from homogenized tissues, and 2) that nucleic acids may quench the fluorescence of CH.

Reports of isolation from tissue, treated with CH, of CH strongly bound to DNA (Weist and Heidelberger, 1953b and c; Heidelberger and Davenport, 1961; Brookes and Lawley, 1964) and RNA (Heidelberger and Davenport, 1961; Boyland and Green, 1962b; Brookes and Lawley, 1964) have been supported, in part, by the demonstrations of CH-binding to both DNA and RNA under test tube conditions (Booth

and Boyland, 1953; Boyland and Green, 1962b; Liquori et al., 1962; Gemant, 1964; Boyland and Green, 1964). The validity of these findings has been challenged (Giovanella et al., 1964; Heidelberger, 1964). Nevertheless, these findings present the possibility of CH penetration to sites of DNA in the nucleus, a situation which has specifically been held to be unlikely by u-v-fluorescence microscopists (Waddington and Goodhart, 1949; Berg, 1951/1952; Richter, 1951, 1952a and b, 1955; Richter and Saini, 1960; Shires, 1962; Jones, 1962; Harvey et al., 1963).

The findings of the microscopists vs. the findings of the biochemists regarding the intranuclear occurrence of aromatic, polycyclic hydrocarbons would seem to be explained by Boyland and Green's observations (1962b) that the fluorescence of CH was quenched by binding with DNA. Thus, the apparent lack of demonstrable CH in nuclear structures observed with u-v-fluorescence microscopy might be interpreted as a consequence of nucleic acid-induced quenching of the fluorescence of intranuclear CH. Similar interpretations of the lack of intranuclear fluorescence of stilbamidine or acriflavine were made when it was learned that both could be or were bound to nucleic acid (Snapper et al., 1951; Tubbs et al., 1964). Several considerations made acceptance of such an interpretation not immediately tenable: 1) It is controversial whether true binding of CH to nucleic acids occurs. 2) Observations by Boyland and Green (1962b) have shown that the presence of salts (e.g. NaCl) at even very low ionic strengths significantly reduce the quenching effect of DNA on

CH fluorescence. 3) Observations have repeatedly been made that binding or association of CH with protein (Miller, 1951; Moodie et al., 1954; Woodhouse, 1954, 1955; Carruthers et al., 1957; Stauf et al., 1960) and purine analogs (Weil-Malherbe, 1946; Waddington and Goodhart, 1949; Berg 1951/1952) does not necessarily result in the quenching of CH fluorescence.

In order to study the effect of nucleic acid on the intracellular distribution of BP, two basically different objective approaches were followed: a) One was to determine the effect of nucleic acid extraction on the intracellular distribution of BP in fixed L-929 fibroblasts and in isolated liver nuclei. b) The other was to determine the distribution patterns of BP in intact living hepatic cells following the experimental relocation and stratification of nucleic acid-rich protoplasmic structures by ultracentrifugation.

Fixation is a prerequisite for solvent or enzymatic removal of nucleic acid from whole cells. EtOH-HOAc combinations have been generally favored fixatives for DNAase (Stowell, 1946; Stein and Gerade, 1950; Korsin, 1951; Jackson and Dessau, 1955; Daoust and Clermont, 1955), for RNAase (Kaufman et al., 1951; Brachet, 1953; Swift, 1955; Nair, 1958; Barka and Dallner, 1959; Pearse, 1960), and for NaCl and TCA extractions (Ris and Mirsky, 1946; Opie and Lavin, 1946; Kaufman et al., 1951; Pearse, 1960). In this study (Chapter V) data on the influence of a number of fixatives on the intracellular pattern of BP showed that HCHO and Zenker's fluid, but not EtOH, preserved BP patterns comparable with those occurring in

living cells. Neither HCHO or Zenker's fluid fixation have been universally recommended for salt extraction of nucleic acids by Pearse (1960), or for DNAase treatment by Stowell (1946), Swift (1955) or Pearse (1960), or for RNAase action on cells (Stowell and Zorzoli, 1947; Lagerstedt, 1956/1957; Nair, 1958). On the basis of other reports that HCHO or Zenker's fluid fixation were compatible with successful use of, at least, the nucleases (Brachet, 1953; Swift, 1955; Sandritter et al., 1957; Pearse, 1960; LeDuc and Bernhard, 1961; Bernhard and Tournier, 1962), nucleic acid extractions were made on HCHO and Zenker-fixed cells and tissue sections.

The retention of nucleic acid by cells submitted to nuclease digestion is one of two major problems encountered in enzymatic digestion of any preparation, fixed or not. Ficq and Errera (1958) have reported loss of less than one quarter of the total DNA from whole liver cells, fixed with HOAc-EtOH or Zenker's fluid. Studies on isolated thymocyte nuclei have variously reported retention of about one fourth of the nuclear DNA after DNAase treatment (Allfrey et al., 1957; Allfrey and Mirsky, 1958; Naora et al., 1961). These studies suggest a certain DNA fraction is inaccessible to phosphodiesterase action. RNA removal is apparently more difficult, the nucleic acid remaining after exhaustive enzymatic and salt extraction (Georgiev, 1958; Zbarski and Georgiev, 1959; Smetana et al., 1963; Steel and Busch, 1963).

A second major problem in DNAase hydrolysis of whole cells or nuclei is assay of the extent of DNA removal. Of the two

histochemical procedures having high specificity for DNA, methyl green staining and the Feulgen reaction (Dische, 1955; Pearse, 1960), the methyl green procedure was considered unacceptable because of its reported anomalous staining of nuclei following various nuclear extraction procedures (Kaufman et al., 1951, 1960; Bloch and Godman, 1955a and b). While the Feulgen reaction was used in this study, it also has the drawback of a relatively high threshold of sensitivity (Widström, 1928; Caspersson, 1932; Ris and Mirsky, 1949; Dische, 1955). A partial explanation for this may be the loss of DNA (up to 8% of the total concentration) during the 1N HCl hydrolysis at 60°C used in the standard Feulgen procedure (Sibatani and Fukuda, 1953). Under the conditions of the present study the Feulgen reaction was thus probably only partially reliable in indicating the absence of DNA after treatment by enzymes with/without subsequent extraction.

Displacement of nuclear chromatin has been accomplished by many authors in a wide variety of living vertebrate cell types (Beams and King, 1934a and b, 1935, 1936; Dornfeld, 1936, 1937; Lucas, 1936; McDougald et al., 1937; Hellbaum, 1936; Guyer and Claus, 1936a and b; Claude, 1943; Waddington and Goodhart, 1949; Bessis, 1950, 1955, 1956; Mateyko and Kopec, 1954a, b and c, 1963; Brenner, 1953; Bernhard et al., 1954; Kopec, 1953, 1955; Mateyko, 1957; Beams et al., 1960; Beams and Kessel, 1962). Cytoplasmic nucleic acids have been reported to stratify discretely in liver cells (Claude, 1943; Lagerstedt, 1948; Bernhard et al., 1954). It has been reported that chromatin associated with the nuclear membrane does not move with the

remainder of the nuclear chromatin, but remains *in situ* (Brenner, 1953). It is uncertain how the failure of the DNA-containing material to sediment with the rest of the chromatin might effect the outcome of this experiment.

With these limitations of the experiments in mind, it is hoped that use of unfixed nuclei may serve as a kind of control over the results using fixed nuclei, that use of ultracentrifugal displacement of nucleic acids may control the limitations in extracting nucleic acids and that removal of nucleic acids from fixed whole cells and from isolated nuclei might function as control over the unsedimentability of some nucleic acid in liver cells.

Materials and Methods

Relative to the Effects of Ultracentrifugal Displacement of Nucleic Acids in Liver Cells

Studies to determine intracellular localization of BP in rat liver parenchymal cells after displacement of cytoplasmic and nuclear components by high centrifugal forces were carried out using an air-turbine ultracentrifuge. The general course of preparation involved 1) ultracentrifugation of liver tissues, 2) mechanical dissociation of cells from centrifuged tissue, and 3) the microscopic examination of the dissociated centrifuged cells in wet *in vitro* slide preparations. In order to confirm displacement of nucleic acids and other hepatic cell components, several routine histologic and histochemical procedures were carried out.

Instrumentation. The air-turbine centrifuge used in this study

was fabricated in the Physical Laboratory of the University of Virginia, Charlottesville, Virginia, in conformity with the general designs of J. W. Beams (1930, 1931, and 1933) and Beams and Pickles (1935). Calibration curves for the G forces developed at given pressure and revolutions per second were drawn for the rotors of this centrifuge by Fritz Linke in the same laboratory. A high-capacity constant-pressure air supply was provided by a Quincy Compressor. The distal end of the air line contained Airflo air filter (Commercial Filters Incorp.) and a DeVilbriss pressure gauge with manual valve.

Preparation and centrifugation of tissues. King-Holtzman rats were sacrificed by subluxation of the cervical vertebrae, the abdominal cavity opened and small fragments of liver were excised and added to a cold complete growth medium (BME or MEM) in a clean petri dish. There, tissue fragments were reduced in size to approximately 0.3 to 0.5 cm. by 1 to 2 cm. long.

The aluminum rotors, which were physiologically clean, were filled with 0.5 to 0.8 cc. of MEM (Microbiological Associates, Incorp) or BME (Microbiological Associates, Incorp.). Three to five liver fragments were placed inside the rotor chamber with the medium and the top screwed into place.

The rotors containing the living liver cells were spun at speeds sufficient to develop a centrifugal force of 400,000 X G (2,500 rpms) for a period of two hours. A few other runs were made at slower speeds (producing 300,000 X G) or for shorter periods of time (1-11/2 hours).

Histologic and histochemical procedures. Routine histologic and histochemical procedures were carried out on paraffin sections of ultracentrifuged liver fixed for one hour in Lillie's HCHO, which included Lillie-Meyer's Hematoxylin and Eosin (Gridley, 1960); the Feulgen reaction (Feulgen and Rossenbeck, 1924; Bauer, 1932), using Leucofuchsin prepared by the method of DeThomasi (1936) and Coleman (1938); staining with Gallocyanin-Chrom Alum (Einarson, 1951) with DNAase and RNAase controls carried out as will be described below; staining with Bromphenol Blue for protein (Mazia et al., 1953); the Bests Carmine procedure with Diastase controls (Lillie, 1948); and the periodic acid-Schiff procedure (PAS) (McManus, 1948) with Diastase controls (Lillie, 1948).

Relative to the Removal of Nucleic Acid from L-929 Fibroblasts

Studies to determine the effect of nucleic acid on the intracellular distribution of CH in L-929 fibroblasts by their removal involved the following procedures: 1) fixation of coverslip cultures with 95% EtOH, 5% TCA, Lillie's HCHO or Zenker's fluid for one hour by methods previously described (the Zenker's-fixed cells were routinely post-treated with Lugol's solution and thiosulfate by the methods described above); 2) removal of nucleic acid from the fixed cells by non-specific extraction with 5% TCA or 1M NaCl or by digestion of the cells with deoxyribonuclease (DNAase) or ribonuclease (RNAase), singly or sequentially; 3) exposure of the treated cells to crystalline beds

of BP by procedures previously described or submission of treated cells to routine cytochemical assays of the extent of nucleic acid removal.

Extraction of Nucleic acid with TCA. Five per cent TCA was used as a combined fixing and extracting agent (Schneider, 1945; Pollister and Ris, 1947). L-cells on coverslips were immersed directly into 5% TCA, and, with hourly changes of solution, were extracted in 5% TCA at 60°C after Kelley and Carlson (1963) for 15 minutes, for 15 minutes at 90°C (Schneider, 1945; Pollister and Ris, 1947), or for 1 , 3, 12, 24 hours at room temperature (approximately 25°C). At the termination of extraction, the cells were thoroughly rinsed with water and mounted in water on crystalline beds of BP. Control cells were mounted on clean glass slides after TCA treatment.

Extraction of nucleic acids with NaCl. Molar NaCl was used after fixation with 95% EtOH (for 15 minutes), Lillie's HCHO (for one hour) or Zenker's fluid (one hour) with post-treatment of Lugol's solution and thiosulfate. Extraction was carried out in a sealed Columbia staining dish at 60°C for 2, 12, 24, and 36 hours (Pearse, 1960).

Removal of nucleic acids by enzymatic digestion. Enzymatic removal of nucleic acids from L-strain fibroblasts was accomplished by incubating the cells in a solution containing a phosphodiesterase, suitable buffers and any required cofactors. Incubation took place in a Columbia staining dish sealed with Silicone grease (Dow Corning). All glassware was de-ionized with concentrated nitric acid and copiously rinsed in triple distilled water (Cutter).

Use of deoxyribonuclease. Substrate specific deoyxribonuclease

(DNAase-Worthington, 3X crystallized) was incubated in 0.1M McIlvaine's buffer at pH 6.0 (Kunitz, 1950; Korsin, 1951; Daoust and Clermont, 1955) with 0.005M of the cofactor magnesium ion (as $MgCl_2$-Kunitz, 1950) or 0.003M $MgCl_2$ (Kaufman et al., 1951) at 37°C for periods of three to 6 hours. The enzyme concentrations used were 0.2, 0.6, 1.0, and 2.0 mg/ml, and with the higher concentrations 1 mg% chloramphenicol (Parke-Davis) was sometimes added (Bulmer, 1965).

DNAase hydrolysis was carried out on cells frozen in isopentane vapor chilled to -196°C by liquid nitrogen (Bloom et al., 1954) followed by fixation in Lillie's HCHO, and on cells fixed directly in Zenker's fluid for one hour, post-treated with Lugol's solution and thiosulfate.

Enzymatic action was stopped by washing the cells with distilled water. For observation, the digested cells were mounted in distilled water on crystalline beds of BP or on clean glass slides.

Two controls were simultaneously run with DNAase hydrolysis. In the first, $MgCl_2$ was omitted from the incubation medium, and in the second, the enzyme was omitted. Both controls were incubated at 37°C for the same duration of time as the complete preparations. The cells were mounted on BP beds or clean glass slides in the same manner as the fully hydrolyzed cells.

Use of ribonuclease. The method of ribonuclease (RNAase) treatment was a modification of the procedure of Brachet (1953). The cells on coverslips were incubated one to two hours at 37°C or 60°C in triple distilled water (Cutter) whose pH had been adjusted to 6.0 with

0.1N NaOH. Enzyme concentration was 0.1 mg/ml of RNAase (CalBio, 3X crystallized, bovine pancreatic source). Treatment of the cells prior to enzymatic hydrolysis included freezing in isopentane vapors chilled to -196°C with liquid nitrogen followed or not followed with fixation in Lillie's-HCHO, and direct fixation in Zenker's fluid for one hour with conventional Lugol's solution-thiosulfate post-treatment. The simultaneously run controls involved incubation in medium not containing RNAase. Both the control and experimental preparations were washed in distilled water and mounted on beds of BP or on clean glass slides for observation with integrated phase-contrast and u-v-fluorescence microscopy.

Cytochemical assay of extraction of nucleic acids. L-fibroblasts treated with non-specific nucleic acid extraction procedures (e.g. NaCl or TCA extraction procedures), or with the phosphodiesterases and their controls were routinely submitted to histochemical tests for nucleic acid. These included: the Feulgen reaction (Feulgen and Rossenbeck, 1924; Bauer, 1932) using Leucofuchsin made according to DeThomasi (1936) and Coleman (1938), and staining with Gallocyanin-Chrom Alum, prepared and applied according to Einarson (1951).

Relative to the Removal of Nucleic Acid from Isolated Liver Nuclei

The general outline of the procedure used in this study of the influence of nucleic acids on the CH distribution relative to isolated liver nuclei is shown in the Flow Chart (Table IV). The methods for isolation of liver nuclei in 0.25M sucrose-$CaCl_2$ and their resuspension

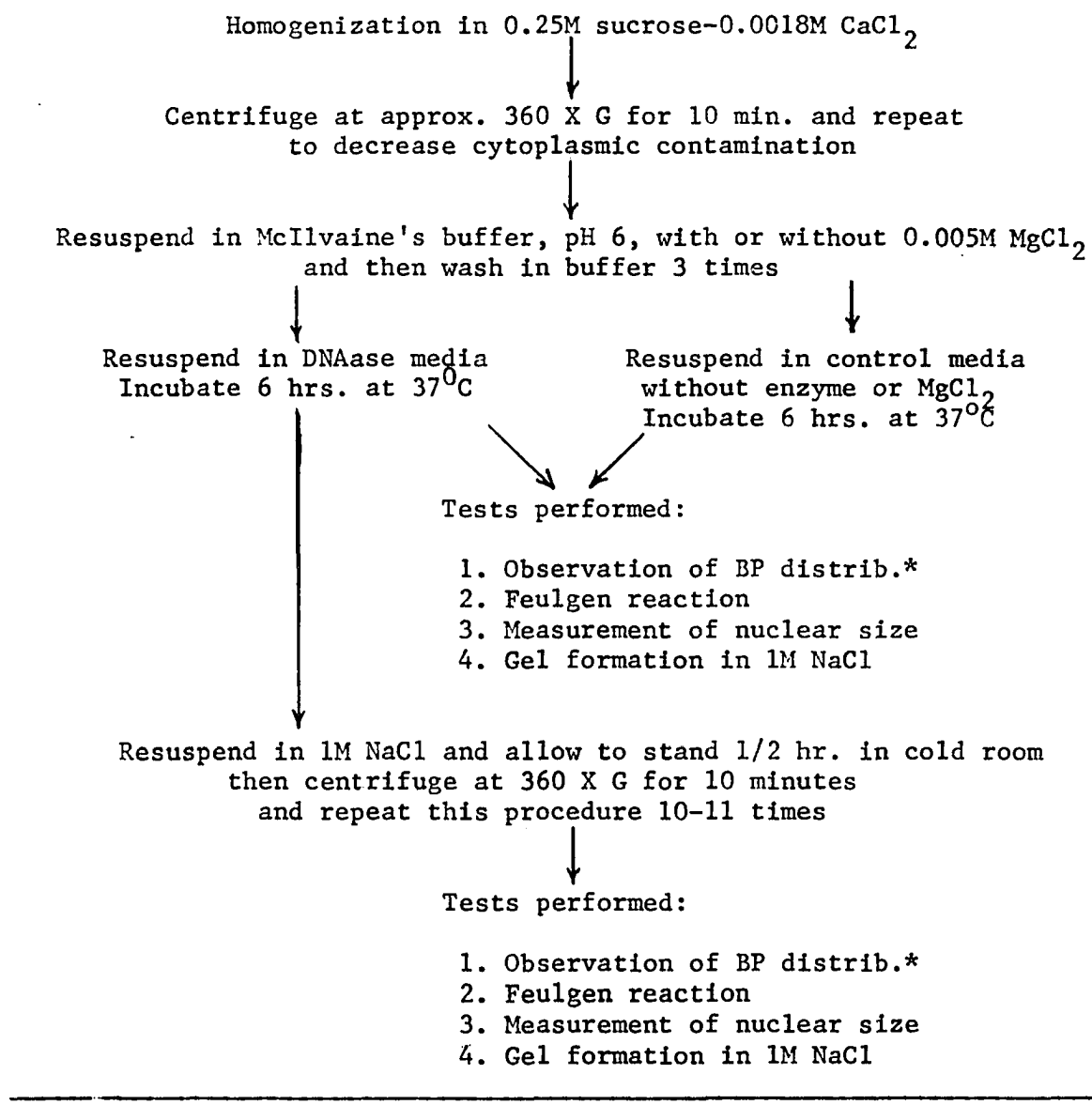
in McIlvaine's buffer have been previously described in Chapter VI.

Nucleic acid extraction procedures. In a modification of methods by Kunitz (1950), Korsin (1951), Daoust and Clermont (1955) and Naora et al., (1961), liver nuclei isolated in 0.25M sucrose-$CaCl_2$ were washed three times in McIlvaine's buffer (pH 6.0) and resuspended in an incubation medium containing a) pH 6.0 McIlvaine's buffer (0.2M dibasic phosphate and 0.1M citric acid), b) 0.005M $MgCl_2$ and c) 1 mg/ml DNAase I (Worthington, 3X crystallized). 10cc of incubation medium was used to digest 1 cc of nuclear fraction for 6 hours at 37°C. For controls, a) nuclei were incubated in media without the enzyme for 6 hours at 37°C and b) nuclei were incubated in McIlvaine's buffer alone at 37°C for 6 hours with or without $MgCl_2$ (0.005M).

To insure complete extraction of nucleic acid, a modification of the method of Georgiev and Chentsov (1962) was used in which isolated nuclei were first subjected to DNAase by the method outlined above followed by NaCl extraction. Nuclei treated with DNAase were suspended in 1M NaCl, centrifuged at 260 X G for 10 minutes and the supernatant decanted. The nuclear pellets were resuspended and extracted in 1M NaCl at refrigerator temperatures (approx. 5°C). At thirty minute intervals the nuclei were centrifuged, supernatant discarded and the nuclear pellets resuspended in 1M NaCl. This procedure was repeated 9-11 times. The full sequence of steps used in the preparation of these DNAase-digested, salt-extracted nuclei is shown in the Flow Chart (Table IV).

TABLE IV

FLOW CHART



* Controls for u-v-fluorescence observation of cells on BP beds were also run using cells placed on clean glass slides.

The precautions concerning the cleaning of glassware and the maintenance of cold temperatures at every step in the procedure (except for the incubation at 37°C) were carried out as described previously.

Routine determinations made on DNA-extracted nuclei. As shown on the Flow Chart (Table IV), control and DNAase-digested isolated liver nuclei and nuclei sequentially treated with DNAase and NaCl were routinely observed by u-v-fluorescence and phase-contrast microscopy to determine the pattern of nuclear uptake of BP. In addition, the following specific determinations were made:

1) Ability of nuclei to form gels. As previously described, the procedure of Dounce (1955) was used to determine the polymeric state of nucleoprotein in treated nuclei.

2) pH measurement. pH measurement was carried out by procedures previously described on liver nuclei treated for removal of nucleic acids.

3) Measurement of nuclear size. In order to quantitate size changes after nucleic acid removal, treated nuclei were placed on a clean glass slide and sealed under a coverslip. Measurement was made of the long and short axis of an individual nucleus three times, and the average length and width derived. All measurements were made at 970X magnification with a Leitz Objeckmikrometer. Measurements on a particular in vitro preparation were terminated after the slide was 20 minutes old.

4) The Feulgen reaction. All experimental and control preparations of liver nuclei were tested for the presence of DNA by the

Feulgen reaction. The procedure used was a modification of that by Hutchison et al., (1958). Smears of the various nuclear pellets were air-dried, fixed in Lillie's-HCHO, hydrolyzed in 1N HCl for 4 minutes and treated with decolorized Schiff's reagent, prepared by the methods of DeThomasi (1936) and Coleman (1938).

Observations

The Intracellular Distribution of BP in Fibroblasts Treated for Removal of Nucleic Acids.

Of the non-enzymatic methods for removing nucleic acids from whole L-fibroblasts (i.e. salt or TCA extractions), only one consistently succeeded in removing all Feulgen-positive material: that using 95% EtOH fixation followed by 2 1/2 hours in 1M NaCl at 60°C. Cells treated in this manner showed a finely granular cytoplasm and a nucleus, containing nucleoli and a precipitated nucleoplasm (Figs. 129) closely resembled unextracted EtOH-fixed cells (Figs. 109, 111, 112, 114, 116). No apparent loss of intranuclear material as a result of salt extraction was detected with phase-contrast microscopy.

EtOH-fixed and salt-extracted fibroblasts exposed to crystalline beds of BP and observed with u-v-fluorescence microscopy displayed an apparently low affinity for BP. There were no specific accumulations of BP in the cytoplasmic components which exceeded the accumulation by the cytoplasmic matrix. Irregular vacuolar spaces distributed at random throughout the cytoplasm displayed relatively lower BP uptakes (Fig. 130). As in the case of unextracted, EtOH-fixed cells (Figs.

110, 113, 115) demonstrable intranuclear uptake of BP was located in the nucleoli of salt-extracted cells (Fig. 130), but the remainder of the nucleus, including the nuclear membrane and the precipitated nucleoplasm, displayed no apparent accumulation of BP.

Fixation and extraction in 5% trichloracetic acid (TCA) at a) room temperature for 1 1/2 hours and b) at 60°C for 5 hours failed to remove Feulgen-stainable material from fibroblasts. Both procedures left a finely precipitated cytoplasm and nucleoplasm. At the lower procedural temperatures, Golgi granules and vacuolar structures were observed, and in the fibroblasts treated at the higher temperatures vacuolated nucleoli and distinct nuclear membranes were observed (Figs. 128, 131). BP was accumulated in relatively high concentrations in Golgi granules; there was no demonstrable BP present in the cytoplasmic vacuoles or any nuclear structure in fibroblasts extracted with both procedures (Figs. 127, 132).

DNAase digestion of frozen and HCHO-fixed L-929 fibroblasts was generally unsuccessful in removing Feulgen-staining material. DNAase treatment of cells frozen in isopentane cooled to -196°C with liquid nitrogen and fixed with Lillie's buffered HCHO yielded cells which contained Feulgen-positive nuclei and which, after exposure to crystalline beds of BP (Fig. 144), contained BP patterns essentially similar to unhydrolyzed cells (Figs. 52, 54, 55, and 58). Cells frozen more slowly at -95°C, post-fixed with Lillie's buffered HCHO and digested with DNAase had badly ruptured cytoplasm and nuclei (Fig. 125). No accumulation of BP was demonstrable in any structure of

the ruptured or partially isolated nuclei of these preparations (Fig. 126).

HCHO-fixation was found usable for RNAase abolition of Gallocyanin-Chrom Alum-staining cytoplasmic material and reduction in the intensity of nuclear staining. Cells frozen in isopentane and post-fixed in Lillie's buffered HCHO showed dark, often refractile, nuclei which contained a granular non-nucleolar karyoplasm not encountered in undigested cells (Fig. 143 vs. Figs. 52, 54, 55, 58). The cytoplasm contained the same fibrous material of undigested HCHO-fixed cells, some centrally located vacuolar structures and a number of randomly dispersed granules of uncertain identity embedded in a hyaline cytoplasmic matrix (Fig. 143). After exposure to BP, the nuclei of these RNAase-extracted fibroblasts showed no affinity for the carcinogen or its fluorescent derivatives (Fig. 144). Cytoplasmic vacuolar structures showed relatively lower BP uptake of CH than the general cytoplasmic matrix (Fig. 144).

Zenker-fixation followed by Lugol's solution and thiosulfate post-treatment proved satisfactory for the removal of nucleic acids by both RNAase and DNAase. The RNAase procedure of Brachet (1953) produced the desired loss of all apparent Gallocyanin-Chrom Alum-staining material from the cytoplasm and from the general karyoplasmic region, excluding chromatin and mitotic chromosomes. Control preparations incubated without the enzyme showed cytoplasmic staining with Gallocyanin-Chrom Alum. Under the phase-contrast microscope, the RNAase-treated cells closely resembled cells fixed in Zenker's fluid with or

without post-treatment for removal of mercury (Figs. 73, 77, 80, 81, 83).

DNAase incubation of L-fibroblasts under the following conditions removed all Feulgen-staining material:

2 mg/ml DNAase with 0.005M $MgCl_2$ and chloramphenicol (Figs. 147, 152, 153, 156),

2 mg/ml DNAase with 0.005M $MgCl_2$ (Figs. 157),

2 mg/ml DNAase and chloramphenicol (Figs. 146).

Zenker-fixed fibroblasts, post-treated for the removal of mercury and incubated in the control solution without enzyme, showed Feulgen staining of chromatin material and chromosomes (Fig. 150). Gallocyanin-Chrom Alum-staining of DNAase-digested fibroblasts presented a staining picture essentially similar to the undigested cells, which is in agreement with the results of other histochemists (Bloch and Godman, 1955a and b; Barka and Dallner, 1959). The phase-contrast microscopic appearance of cells treated with the various DNAase digestion methods (Figs. 146, 147, 152, 153, 156, 157) and that of cells incubated without the enzyme (Fig. 150) were similar to that of Zenker-fixed cells with or without mercury (Figs. 73, 77, 80, 81, 83).

The BP patterns within Zenker-fixed L-929 fibroblasts treated with RNAase (Figs. 134, 135, 137, 140, 142) or with the various DNAase methods, with (Figs. 145, 148, 151, 154, 158) or without enzyme (Fig. 149), were essentially similar. All nuclear and chromosomal structures including nuclear membranes, nucleoli, interphase non-nucleolar karyoplasm, metaphase and anaphase chromosomes, and telophase karyomeres

displayed no apparent accumulation of BP after the DNA (Figs. 145, 148, 151, 154, 155, 158, 160) or RNA (Figs. 134, 135, 137, 140) removal. Golgi granules and Golgi zones showed no relatively higher BP concentrations than those in the general cytoplasmic matrix, while cytoplasmic vacuoles showed relatively lower BP uptake than the matrix (Figs. 134, 135, 137, 140, 145, 148, 151, 154, 155, 158, 160). Interzonal spindles in both DNAase-digested (Fig. 137) and RNAase-digested cells (Fig. 151) showed relatively lower BP uptake in early anaphase, while in later anaphase or early telophase, the spindles showed BP uptake comparable to that of the cytoplasmic matrix (Figs. 140, 142, 154). The pattern of intracellular BP in enzymatically treated fibroblasts was the same as that in Zenker-fixed cells, with or without mercury (Figs. 74, 76, 78, 79, 82).

L-929 fibroblasts fixed with Zenker's fluid, post-treated with Lugol's solution and thiosulfate and successively digested with DNAase and RNAase, showed no color after the Feulgen reaction and a light unlocalized blue color after Gallocyanin-Chrom Alum staining. The phase-contrast microscopic appearance of these cells (Figs. 161, 163) was similar to that of cells treated individually with RNAase (Figs. 133, 136, 138, 139, 141) or with DNAase (Figs. 146, 147, 152, 153, 156, 157). The intracellular distribution of BP in cells treated successively with DNAase and RNAase was the same as that encountered in cells treated individually with the enzymes (Figs. 162, 164 vs. 134, 135, 137, 140, 142, 145, 148, 151, 154, 155, 158).

The Intracellular Distribution of BP in Ultracentrifuged Liver Cells

Centrifuged liver cells, observed with phase-contrast microscopy contained at least four more or less distinct strata of cellular components. They were, in order of centripetal to centrifugal displacement: 1) a layer of highly refractile lipid droplets (Figs. 244, 246); 2) a hyaloplasmic layer with indistinct vacuoles and strands (Fig. 246); 3) a complex layer containing the whole nucleus and numerous granules and vesicles, apparently constituents of the chondriome (Figs. 244, 246, 248); and 4) an distinct layer of fine granules and strands, embedded in a hyaloplasmic matrix (Figs. 244, 246, 248). Varying degrees of overlapping of adjacent strata occurred in all observed cells, probably due in part to the preparative treatment endured by the viable, ultracentrifuged liver cells (Mateyko and Kopec, 1963).

Of the two layers situated at the centripetal end of the ultracentrifuged hepatocyte, the weakly eosinophilic lipid stratum (Fig. 239) was the most easily recognized and frequently encountered in living preparations. As previously reported (Beams and King, 1934b; Claude, 1943; Bernhard et al., 1954; Kopec, 1955), and contrary to Lagerstedt (1949), neither layer included basophilic (Fig. 139) or Gallocyanin-stainable nuclei acid (Fig. 243). The presence of formed inclusions in the second layer, unreported by previous observers of stratified rodent and amphibian liver cells (Claude, 1943; Lagerstedt, 1949; Bernhard et al., 1954; Kopec, 1955), may have been contaminating components from other strata.

The mitochondrial and fine granule strata at the centrifugal

end of stratified hepatocytes observed with phase-contrast microscopy exhibited roughly similar nucleic acid and carbohydrate histochemistry in three different preparations. Basophilia (Fig. 239), RNAase-extractable Gallocyanin-stainable material (RNA) (Figs. 240, 243), and PAS and Best's Carmine-staining material (the latter test being prevented by Diastase pretreatment) were continuous throughout both strata, contrary to Claude (1943), Lagerstedt (1949), Bernhard et al., (1954) and Kopec (1955) who all described separation of a more centrifugally located stratum of carbohydrate from a relatively less dense nucleic acid and mitochondrial layer. These workers utilized lower centrifugal forces or shorter durations of centrifugation than were employed in the present study.

The presumptive Golgi complex and the small granule component of hepatic cells were not consistently located in any one stratum after ultracentrifugation. Complexes of strands and granules similar to those observed in uncentrifuged hepatocytes and tentatively identified as the Golgi complex, were found at the levels of the 2nd (Fig. 246), 3rd (Fig. 246) and the 4th strata (Fig. 248). The small phase-dark granule component was also randomly located in the stratified cytoplasm in all preparations observed (Figs. 244, 248). No previous report of the deposition of the Golgi complex in ultracentrifuged liver cells has been apparently made, but Beams (1951) noted that the Golgi complex of most cell types migrates toward the centripetal pole due to its high lipid content.

As previously described for ultracentrifuged cells observed in

fixed preparations (Beams and King, 1943b; Claude, 1943; Brenner, 1953) or in living preparations (Kopec, 1955), most of the intracellular material in viable hepatocytes was found sedimented against the centrifugal pole of the nucleus (Figs. 244, 246, 248). The remainder of the nucleus appeared clear except for fine threads connecting the sedimented mass with the centripetal portions of the nuclear rim (Figs. 244, 246) (Claude, 1943; Lagerstedt, 1949; Brenner, 1953). Nucleic acid histochemistry revealed a third component in ultracentrifuged nuclei: a nuclear rim containing Feulgen positive, Gallocyanin-stainable material which was apparently only partially removed either with DNAase or RNAase digestion (Figs. 240-243). The rim was continuous around the entire perimeter of the centrifuged nucleus and may be interpreted as nuclear membrane-associated heterochromatin (Brenner, 1953). Nucleoli were obscured as structural or histochemical entities in the heavy chromatin sediment at the centrifugal nuclear pole.

The intracellular pattern of BP uptake by ultracentrifuged hepatic parenchymal cells as observed with u-v-fluorescence microscopy included a differential accumulation of the CH in the cytoplasm but no demonstrable BP uptake by any nuclear structure.

The highest BP concentrations in the cytoplasm were localized in the lipid droplets of the first layer, while slightly lower relative accumulations occurred in the unstratified Golgi complex and the small granule component (Figs. 245, 247, 249). In general, BP distribution in the cytoplasmic matrix seemed to be invariable, with two exceptions: 1) BP appeared to accumulate in relatively higher concentrations in the

centrifugal border of the 4th stratum (Fig. 247), and 2) relatively lower BP accumulations appeared in the general region of the second stratum (Figs. 247, 249). No demonstrable BP uptake was discernible in mitochondria of the third stratum (Figs. 247, 249) or in vacuolar structures of the second stratum (Fig. 247).

The stratified karyoplasm showed no demonstrable BP accumulation in the nucleic acid-rich centrifugal chromatin mass, the chromatin threads of the nuclear rim or in the nucleic acid-poor unstratified karyoplasmic regions (Figs. 245, 247, 249).

The Distribution of BP in Isolated Nuclei From Which Nucleic Acids Had Been Extracted

The liver nuclei treated with DNAase were essentially similar in phase-contrast microscopic appearance to the control nuclei incubated with or without $MgCl_2$. All nuclei contained one to several compact nucleoli in a moderatly dark karyoplasm (Figs. 310, 312, 314, 316, 318, 320, 322, 324, 325). Light vacuolar spaces, perhaps related to the clear regions of DNAase-treated nuclei seen by Anderson (1953b), occurred in the more peripheral karyoplasm of both experimental (Figs. 322, 324, 325) and control nuclei (Figs. 310, 312, 316, 320). Nuclear blebbing was prominent in all nuclei incubated with the enzyme (Figs. 322, 324, 325) or in McIlvaine's buffer, with $MgCl_2$ (Figs. 314, 318, 320) or without $MgCl_2$ (Figs. 310, 312, 316). Some distortion of nuclear shape was incurred by the blebs, but nuclei generally were round to oval in shape, and measurement on their profile diameters showed the average sizes of experimental and control nuclei to be

comparable (Table V). The over-all similarity in appearance of DNAase-treated nuclei with their controls was reminiscent of the results with DNAase-treated fibroblasts. The lack of Feulgen-staining material in the enzyme-treated nuclei constituted the only major difference in the two sets of nuclei isolated in sucrose (Table V).

Liver nuclei digested with DNAase displayed no demonstrable accumulations of CH in any structure located within the boundary of the nuclear rim; the latter showed relatively high concentrations of BP (Figs. 323, 326). Control nuclei, both with or without the addition of $MgCl_2$, showed a similar pattern of BP uptake (Figs. 311, 313, 315, 317, 319, 321).

Liver cell nuclei sequentially treated with DNAase and 1M NaCl were shrunken (Table V), wrinkled and somewhat distorted in shape (Figs. 328, 330, 332, 333, 335). The extraction left a nuclear rim connected to a residual nucleolus by a few strands of dark material (Figs. 328, 330, 332, 333, 335). The composition of these thoroughly nucleic acid-extracted nuclei has been shown to include a small portion of the original ribonucleoprotein, some uncharacterized lipid and an acidic protein (Mirsky and Pollister, 1946; Wang et al., 1953; Georgiev, 1958; Zbarski and Georgiev, 1959; Zbarski and Ermolaeva, 1960; Zbarski et al., 1962; Georgiev and Chentsov, 1962; Steele and Busch, 1963; Busch et al.,; 1963). All chromosomal and perichromosomal globulins, soluble enzymes, two thirds of the ribonucleoprotein and all the DNA with its associated histone and tryptophane-rich proteins are reportedly removed by salt extraction, alone or

TABLE V

CHARACTERISTICS OF NUCLEI FROM WHICH NUCLEIC ACIDS HAVE BEEN REMOVED

Procedure	Feulgen Reaction	Gel	Total Count & Number Preps.*	Diameter ** Long & Short	BP Utake
1. Nuclei isolated in 0.25M sucrose-$CaCl_2$	yes	strong	1453 - 11	11.0 X 8.7	rim only
2. McIlvaine's Buffer, pH 6 incub. 6 hrs. with $MgCl_2$	yes	none	805 - 4	5.7 X 5.2	rim only
3. McIlvaine's Buffer, pH 6 incub. 6 hrs. without $MgCl_2$	yes	none	680 - 4	5.3 X 5.0	rim only
4. DNAase - 6 hrs. at pH 6 in McIlvaine's Buffer with $MgCl_2$	no	none	607 - 6	6.6 X 6.5	rim only
5. DNAase - 6 hrs at pH 6 in Buffer with $MgCl_2$, followed by 1M NaCl 11X	no	none	53 - 5***	3.8 X 3.6	rim only

* The total count is the total number of cells measured. The number of preparations is the number of different preparations of a given procedure on which measurements were made.

** The diameter was measured across the long and short axis of the nuclear profile. The units are micra.

*** The low number of nuclei counted reflects the poor yield from the preparation.

in conjunction with DNAase treatment (Mirsky and Pollister, 1946; Mirsky and Ris, 1949; Georgiev, 1958; Zbarski and Georgiev, 1959; Zbarski et al., 1962; Georgiev and Chentsov, 1962; Steele and Busch, 1963; Busch et al., 1963).

DNAase- and salt-extracted nuclei displayed relatively high accumulations of BP in the nuclear rim but no demonstrable BP uptake by structures within the nucleic acid-extracted nucleus (Figs. 327, 329, 331, 334). The pattern of apparent BP uptake was similar to that observed in nuclei isolated and observed in the various sucrose solutions (Figs. 251, 253, 255, 259, 261), in McIlvaine's buffer and phosphate buffers (Figs. 277, 280, 282, 284, 295, 297, 299, 303, 305, 307, 309), and in the DNAase control procedures involving incubation of nuclei at 37°C for six hours in McIlvaine's buffer at pH 6.0, with or without $MgCl_2$ (Figs. 311, 313, 315, 317, 319, 321), all of which contained material stainable with the Feulgen reaction (Table IV). This pattern of BP uptake was also comparable to that observed in nuclei containing no Feulgen-positive material after DNAase digestion (Figs. 323, 326).

Conclusions

Relative to the Problem of the Nucleic Acid Quenching of CH Fluorescence

The results of these studies involving 1) partial nucleic acid extraction with individual phosphodiesterases, 2) more complete removal of both RNA and DNA enzymatically or by salt extraction, and 3) the

ultracentrifugal preparation of nucleic acid-free regions in the karyoplasm of intact liver cells suggest nucleic acids do not demonstrably influence the apparent intracellular distribution of BP compared with the treated controls. The removal or displacement of nucleic acid from regions rich in these compounds, such as chromatin, nucleoli, nonnucleolar karyoplasm and chromosomal structures, in no case resulted in an increase of BP fluorescence in these regions. If BP had been localized at these nucleic acid-rich sites and its fluorescence had been quenched there, it would be expected that removal of nucleic acid would have resulted in the appearance of BP-fluorescence. The appearance of demonstrable BP fluorescence in nucleoli of EtOH-fixed and NaCl-extracted fibroblasts was part of an intracellular BP pattern which was similar to that in unextracted, EtOH-fixed cells, and so this particular occurrence of intranuclear BP uptake after general nucleic acid removal does not constitute evidence of nucleic acid quenching of BP fluorescence.

An additional general observation of this study was that the over-all BP patterns relative to extracted cells and nuclei and to ultracentrifuged liver cells were the same as those in untreated controls. Relative to all structures within the confines of the nuclear rim, the results of this study show that all the nucleic acid of fibroblasts, liver cells or isolated liver nuclei treated for limited or total nucleic acid extraction or with ultracentrifugation showed no demonstrable uptake of BP. This lack of intranuclear affinity for CH is an established finding in all nucleated living vertebrate cell types.

The absence of fluorescence in nucleic acid-extracted nuclei can be interpreted in two ways in addition to the interpretation that no BP is present at these intranuclear sites. 1) After exhaustive nucleic acid extraction, such as is achieved by the procedure of sequential treatment with DNAase and NaCl extraction of liver nuclei, there may be little material left in which BP could accumulate. Bromphenol Blue staining, size measurement and the electron micrographs of Zbarski et al., (1962) and Georgiev and Chentsov (1962) all attest that isolated nuclei processed with the above extraction procedure contain little material between the visible residual structures. However, isolated nuclei treated with RNAase and DNAase, singly or sequentially, and the non-chromatic karyoplasm in the ultracentrifuged nuclei of liver cells all showed the presence of protein with Bromphenol Blue staining. Thus in these nuclei, material of a protein nature is available for BP localization.

2) A second possible interpretation of the absence of intranuclear fluorescence after nucleic acid extraction or displacement would be that in all of these treated nuclei small amounts of nucleic acid, at undetectable levels, might remain and continue to quench the fluorescence of BP, as in untreated cells. The experimental limitations of this study are, as previously pointed out, such that it is not possible to reliably detect small amounts of nucleic acid.

The evidence from these studies on the influence of nucleic acid on the intracellular distribution of BP does not support the

possibility of an intranuclear quenching of CH fluorescence by nucleic acids. Additional evidence from previous facets of this study also supports this contention that no CH-fluorescence quenching by nucleic acids occurs intracellularly. Citric acid-isolated nuclei and sucrose-isolated nuclei resuspended in acetate buffer, both of which contained Feulgen stainable material, showed demonstrable BP uptake intranuclearly and particularly by apparent nucleic acid-rich chromatin material.

Information from the present study on which to assess the influence of cytoplasmic RNA as a potential quenching agent is limited. No apparent effect of RNA removal from the cytoplasm of L-929 fibroblasts or from the centripetal cytoplasm of centrifuged liver cells on the cytoplasmic distribution of BP was evident. The apparent reduction in BP uptake by the cytoplasm in the second stratum of ultracentrifuged liver cells occurred in a region not containing histochemically demonstrable nucleic acid. This association between demonstrably low levels in BP accumulation and low RNA levels parallels the finding by Jones (1962) that the intracellular accumulation of BP by developing mammalian red cells decreased as the cellular RNA concentration decreased, and it contrasts with the increase in BP uptake by late anaphase interzonal spindles at the time RNA concentrations are increasing, as shown previously. The results of this and previous Chapters and previous literature reports offer no evidence that cytoplasmic RNA is associated with a reduction in the fluorescence of BP.

As has previously been mentioned the nucleus is an especially advantageous structure to study relative to the intracellular distribution of CH. The results of study of the influence of nucleic acids on the pattern of CH in the nucleus have been interpreted as consistent with the view that quenching of intranuclear CH fluorescence by nucleic acids is not the factor responsible for the apparent lack of intranuclear BP uptake. The outcome of such an interpretation requires comparison with the results of other studies in previous Chapters in order to set it in proper perspective.

Berg (1951/1952) considered that the lack of intranuclear uptake of BP was due to an absence of lipid within the nucleus. There are, however, numerous biochemical and cytochemical reports of lipid in the nucleus in general (Stoneburg, 1939; Hack, 1948; Engebring and Laskowsky, 1953; Wang et al., 1953; Dounce, 1955; Chauveau et al., 1956; Levin and Thomas, 1961), in chromosomes - both mitotic and interphase (Claude and Potter, 1943; Ris and Mirsky, 1946; Cohen, 1949; Brock et al., 1952; Idelman, 1957, 1958a and b; Chayen et al., 1957, 1959; LaCour et al., 1958), in nucleoli (Brock et al., 1952; Zagury, 1957; Barer and Dick, 1957; Georgiev, 1958; Zbarski et al., 1962; Georgiev and Chentsov, 1962) and in the nuclear membrane (Schmitt, 1938).

Experimental results of various portions of this report are germane to the problem of understanding the lack of intranuclear uptake of BP in two different aspects, now that nucleic acid is

considered an unimportant factor in the determination of that lack of uptake.

1) The results of experiments described in Chapter VII were interpreted as consonant with Berg's hypothesis that lipid was a factor in determining sites of CH accumulation. If lipid is an intranuclear constituent and if this interpretation of lipid as an influential factor in the intracellular distribution of CH is correct, the intranuclear lipid must be considered in some way structurally inaccessible to CH.

2) Of the various occurrences of demonstrable BP uptake by intranuclear structures in this report, all involved exposure to agents capable of unmasking lipid, with two exceptions. The two exceptions, which remain essentially unexplained, were nucleolar uptake of Berg's BP in lipid extracted cells and the occasional karyoplasmic accumulation of BP in pH 7.4 Na-phosphate nuclei. The unmasking agents which were associated with intranuclear BP uptake were 95% EtOH (Ciaccio, 1926), citric acid (Brock et al., 1952; Ackerman, 1952) and acidified acetate (Cohen, 1949; Ackerman, 1952; LaCour et al., 1958). These agents and other oxidizing organic acids were those used in the histochemical demonstration of intranuclear lipid (Cohen, 1949; Brock et al., 1952; Zagury, 1957; Idelman, 1957, 1958a and b; Chayen et al., 1957, 1958; LaCour et al., 1958). It may be speculated that, contrary to Berg's hypothesis, some but not all forms of intracellular lipid can associate with BP, but that under certain conditions (presence of

unmasking agents), which deserve further study, the availability for association with BP of some of these inaccessible lipids may be increased.

CHAPTER IX

SUMMARY

It is essential to an understanding of the mechanism of chemical carcinogenesis that the intracellular sites of CH-activity in living cells be firmly established. Available information on the intracellular sites of CH-action is incomplete and controversial and has involved use of multiple and very diverse technical approaches capable of introducing alterations in the biochemical content and the physiology of living cells which would, although not previously evaluated, effect spurious patterns of intracellular CH.

This study has centered on 1) the intracellular distribution of CH in specialized living L-929 fibroblasts and rat hepatic parenchymal cells, 2) the evaluation of technical procedural systems used in determining the protoplasmic sites of CH-distribution as related to the live cell patterns of CH distribution, and 3) the influence or role of intracellular lipid, protein and nucleic acid on the u-v-fluorescence pattern of protoplasmic distribution of CH.

I. Studies on the living cell systems warrent the following points:

1) The u-v-fluorescence pattern of intracellular BP in living

L-929 fibroblasts and hepatic parenchymal cells is similar to that in other living nucleated cell types. Elements of the Golgi complex show BP accumulation which is apparently greater than that by the general cytoplasmic matrix, while all nuclear structures, mitochondria and cytoplasmic vesicles show no demonstrable BP accumulation.

2) This pattern of intracellular BP was shown to be unaltered by a) duration of in vitro exposure to BP, b) irradiation with u-v light and c) simultaneous exposure to BP and a cocarcinogenic substance, croton oil.

3) In mitosis of L-929 fibroblasts, a) BP is not accumulated in chromosomal or karyomeric structures at any stage of mitosis; b) The presence or absence of a nuclear membrane in prophase or telophase is not required for maintenance of the characteristic lack of affinity for CH by intranuclear structures; c) The dissociating nuclear membrane of prophase and the reforming nuclear membrane of telophase both show distinct high BP accumulation, a situation not found in the interphase nuclear membranes; d) No demonstrable BP uptake was found in the interzonal spindle until late anaphase and early telophase when both BP and RNA show a parallel increase.

II. Analytic studies of the influence of various chemical and physical components characterizing technical procedures, some of which have been utilized in previous investigations on the sites of intracellular distribution of CH, warrant the following points:

1) Preservation of the live-cell pattern of intracellular CH

distribution is obtainwing cells by three conventional histologic s d five multiple-agent fixatives, to the exteeserve specific cell structures. Preservatllular patterns of BP were not related to a) ixation, b) to the chronology of exposure CH and fixative, and c) to fixation by vaporprior to fixation.

2) An alteratiacellular pattern of BP is occasioned by fixatich Palade's buffered OsO_4.

3) Th characteear accumulation of BP in living cells is pres in various types of sucrose isolation media any of the citric acid isolation media tested.

4) Post-fixatiohydration and clearing results in the marked all intracellular distribution pattern of CH, vment to remove mercury from cells fixed with meves is not associated with any alterations of ne cells.

5) The absence tion of BP in living cells is a characteristin sucrose-isolated nuclei resuspended in Mchosphate buffer, in potassium phosphate buffte buffer at a variety of pHs on the acid such maintenance is

L-929 fibroblasts and hepatic parenchymal cells is similar to that in other living nucleated cell types. Elements of the Golgi complex show BP accumulation which is apparently greater than that by the general cytoplasmic matrix, while all nuclear structures, mitochondria and cytoplasmic vesicles show no demonstrable BP accumulation.

2) This pattern of intracellular BP was shown to be unaltered by a) duration of in vitro exposure to BP, b) irradiation with u-v light and c) simultaneous exposure to BP and a cocarcinogenic substance, croton oil.

3) In mitosis of L-929 fibroblasts, a) BP is not accumulated in chromosomal or karyomeric structures at any stage of mitosis; b) The presence or absence of a nuclear membrane in prophase or telophase is not required for maintenance of the characteristic lack of affinity for CH by intranuclear structures; c) The dissociating nuclear membrane of prophase and the reforming nuclear membrane of telophase both show distinct high BP accumulation, a situation not found in the interphase nuclear membranes; d) No demonstrable BP uptake was found in the interzonal spindle until late anaphase and early telophase when both BP and RNA show a parallel increase.

II. Analytic studies of the influence of various chemical and physical components characterizing technical procedures, some of which have been utilized in previous investigations on the sites of intracellular distribution of CH, warrant the following points:

1) Preservation of the live-cell pattern of intracellular CH

distribution is obtained after fixation of living cells by three conventional histologic single-agent fixatives and five multiple-agent fixatives, to the extent that the fixatives preserve specific cell structures. Preservation of live cell intracellular patterns of BP were not related to a) the length of time of fixation, b) to the chronology of exposure of the living cells to CH and fixative, and c) to fixation by vapors or freezing of cells prior to fixation.

2) An alteration of the live-cell intracellular pattern of BP is occasioned by fixation with 95% EtOH and with Palade's buffered OsO_4.

3) The characteristic lack of intranuclear accumulation of BP in living cells is preserved in nuclei isolated in various types of sucrose isolation media but is not preserved in any of the citric acid isolation media tested.

4) Post-fixation treatment involving dehydration and clearing results in the marked alteration of the live-cell intracellular distribution pattern of CH, while post-fixation treatment to remove mercury from cells fixed with mercury-containing fixatives is not associated with any alterations of the BP pattern within the cells.

5) The absence of intranuclear accumulation of BP in living cells is a characteristic which is maintained in sucrose-isolated nuclei resuspended in McIlvaine's citric acid-phosphate buffer, in potassium phosphate buffer and in sodium phsophate buffer at a variety of pHs on the acid side of pH 7.5, but no such maintenance is

is found in nuclei resuspended in acetate buffers at various pHs.

III. Studies on the influence of intracellular lipid, protein and nucleic acids on the protoplasmic distribution of BP warrant the following points:

1) Extensive removal of lipid from cells grown in vitro on BP beds by $CHCl_3$:MeOH extraction ranging from five to twelve hours (in contrast to a few minutes in the original Folch-Pi method) resulted in the apparently complete loss in fluorescent CH and no apparent retention of BP by residual proteins or other material resistant to the lipid extractions.

2) Evidence consonant with Berg's hypothesis that lipid content of specific cell sites determines uptake of BP by those sites was obtained. The low levels of BP uptake by lipid-extracted cells was possibly related to adsorption phenomena.

3) The absence of any demonstrable intranuclear accumulation of BP in living cells, visualized by fluorescence microscopic methods, is not referable to the quenching of CH-fluorescence by nucleic acids.

IV. On the basis of extensive analytic evidence presented in this study, the u-v-fluorescence microscopic method provides a useful and reliable means of determining the specific sites of CH distribution in both living and technically modified cells.

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APPENDIX

Plate Legend

The following figures are un-retouched phase-contrast and u-v-fluorescence photomicrographs of living cells and ordinary light microscopic photomicrographs of fixed whole and sectioned cells. They were all taken with a 35 mm. Leica camera equipped with a MicroIbso attachment having a 1/3d X conical extension tube.

List of Abbreviations

DMP	dark-medium phase-contrast microscope
UVFM	ultraviolet fluorescence microscope
OLM	ordinary light microscope
B	cytoplasmic bleb
Bac	bacterium
C	chromosomal material
Cp	perichromosomal cytoplasm
Eq	equatorial plate
F	fibrous material
Fp	fine cytoplasmic process
G	Golgi granule
Gz	Golgi zone or Golgi complex

H	chromatin
Hc	chromatin or heterochromatin
I	interzonal spindle region
Ic	intranuclear cytoplasmic tongue
If	interzonal spindle fiber
K	karyomere
Ky	non-nucleolar karyoplasm
L or Ld	frank lipid droplets
M	mitochondria
nc	nucleolus
Mc	nuclear fragment
Nm	nuclear membrane
Pc	cytoplasmic process
R	nuclear rim
Rd	cytoplasmic ridge
Sg	small granule component
V	cytoplasmic vesicle or vacuole
Va	vacuole
Vp	velamentous cell process
X	cell

Explanation of Plates

Plate I

Figure 1. Portions of three L-fibroblasts showing Golgi granules (G), mitochondria (M) and nuclei, containing chromatin (Hc), nucleoli (nc) and nuclear membranes (Nm). The middle cell contains a micronucleus (Mc). DMP 1317X

Figure 2. Same cells as in Figure 1 demonstrating the relatively high BP uptake in Golgi granules (G) and no apparent uptake in the mitochondria (M), micronucleus (Mc) or in any nuclear structure (N). UVFM 1317X

Figure 3. Portions of three L-929 fibroblasts showing BP accumulation in the general cytoplasmic matrix of all cells including the velamentous process of the middle cell (Vp). Relatively high CH uptake occurs in the Golgi granules (G), but relatively little BP has accumulated in a nuclear fragment (Mc) or in any nuclear structure (N). UVFM 1317X

Figure 4. The same cells as in Figure 3 presenting the dark Golgi granules (G), dark nuclear fragment (Mc) and nuclear structures (N) similar to those in Figure 1. The middle cell contains a thin velamentous process (Vp). Fibrous structures (F), perhaps associated with the cell surface, appear as thread-like structures in the thin cytoplasm. DMP 1317X

Figure 5. L-fibroblasts on a BP bed showing relatively high CH uptake in Golgi granules (G) and no demonstrable BP uptake by any

nuclear structure (N) or mitochondria (M). UVFM 1317X

Figure 6. Same L-fibroblasts as in Figure 5 showing an aggregation of Golgi granules (G), some mitochondria (M) and nuclear structures including a nucleolus with a vacuole (nc). DMP 1317X

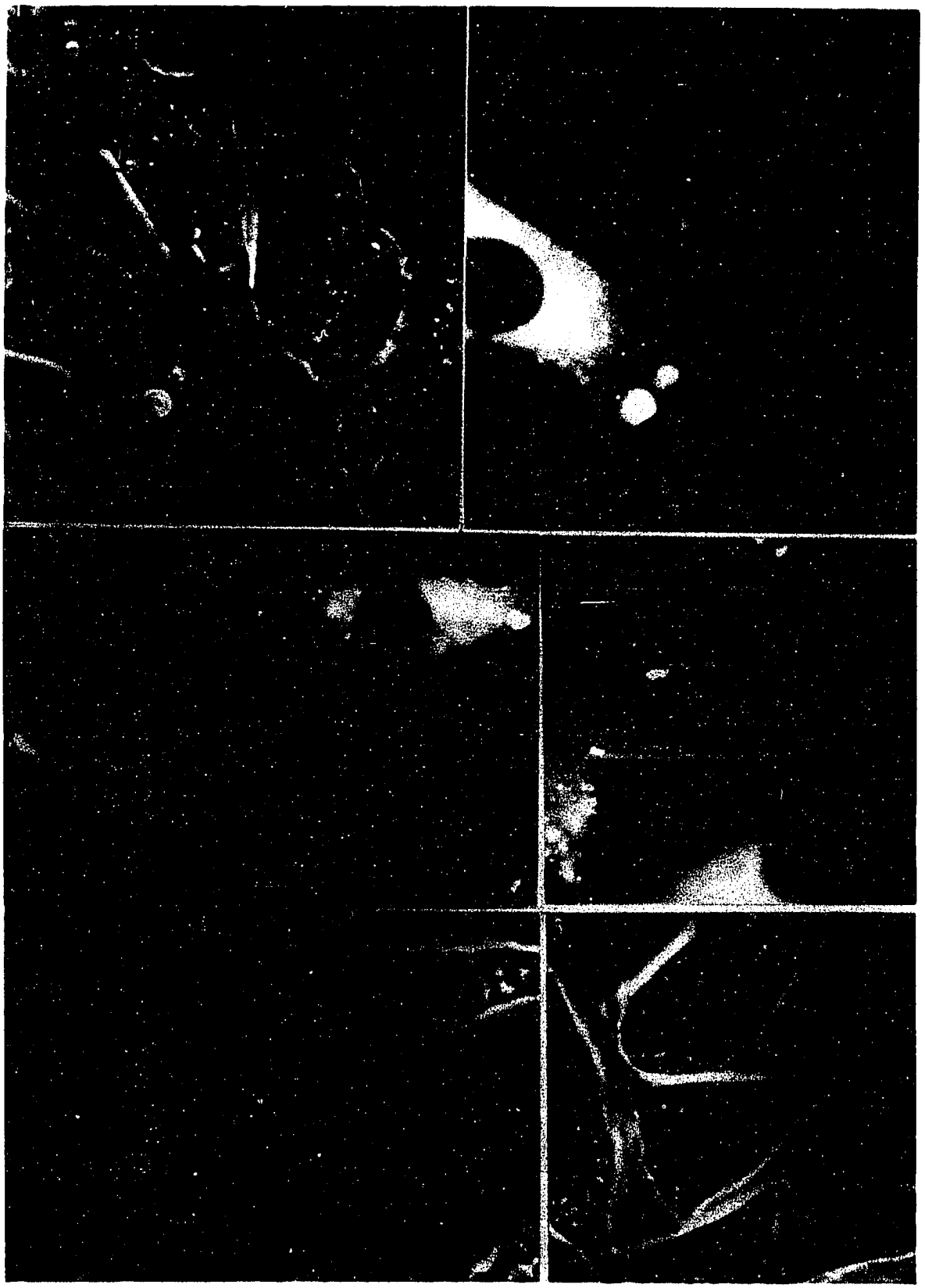

Plate II

Figure 7. L-929 fibroblasts showing many fine cytoplasmic processes (Fp) emanating from the lateral cell margin and many fibrous structures (F) coursing over the thin peripheral cytoplasm. A large juxtanuclear Golgi zone (Gz) surrounds half of the nuclear perimeter. DMP 1317X

Figure 8. Same cell as in Figure 7 but viewed at a slightly higher focal plane showing the same fibrous structures of Figure 7 (F) associated with the cell surface. The extensive Golgi zone (Gz) remains in focus. Visible nuclear structures include strands of granular heterochromatin (Hc). DMP 1317X

Figure 9. Same cell as in Figures 7 and 8 showing relatively high accumulations of apparent BP in the structures of the Golgi zone (Gz). Relatively lower BP accumulations, comparable with uptake by the general cytoplasmic matrix, are seen in the fine cytoplasmic processes (Fp) and in the surface fibrous structures (F). All nuclear structures (N) show no apparent BP uptake. UVFM 1317X

Figure 10. Several L-fibroblasts in broad lateral contact, containing nuclear structure resembles that of Figures 1, 4, 6, 7 & 8, and which includes compact and vacuolated nucleoli (nc). The lower central cell nucleus contains an intranuclear extension of cytoplasm (Ic). DMP 1317X

Figure 11. Same cells as in Figure 10 showing no uptake of demonstrable BP by any nuclear structure (N) except for that observed in an intranuclear extension of the cytoplasm (Ic). UVFM 1317X

Figure 12. L-fibroblasts exposed to continuous u-v light for 10 minutes mounted, while living, on a crystalline bed of BP. Hyaline blebs appear on the cell surfaces (B) but no other change in cytomorphology is apparent. The round central cell is in early anaphase and contains daughter chromosomes (C) and Golgi granules (G). DMP 1317X

Figure 13. Same cell as in Figure 12 showing no apparent BP uptake in any nuclear structure (N), in the internum of cytoplasmic blebs (B) or in anaphase chromosomes (C). Relatively high BP accumulations occur in the Golgi granules. (G). UVFM 1317X

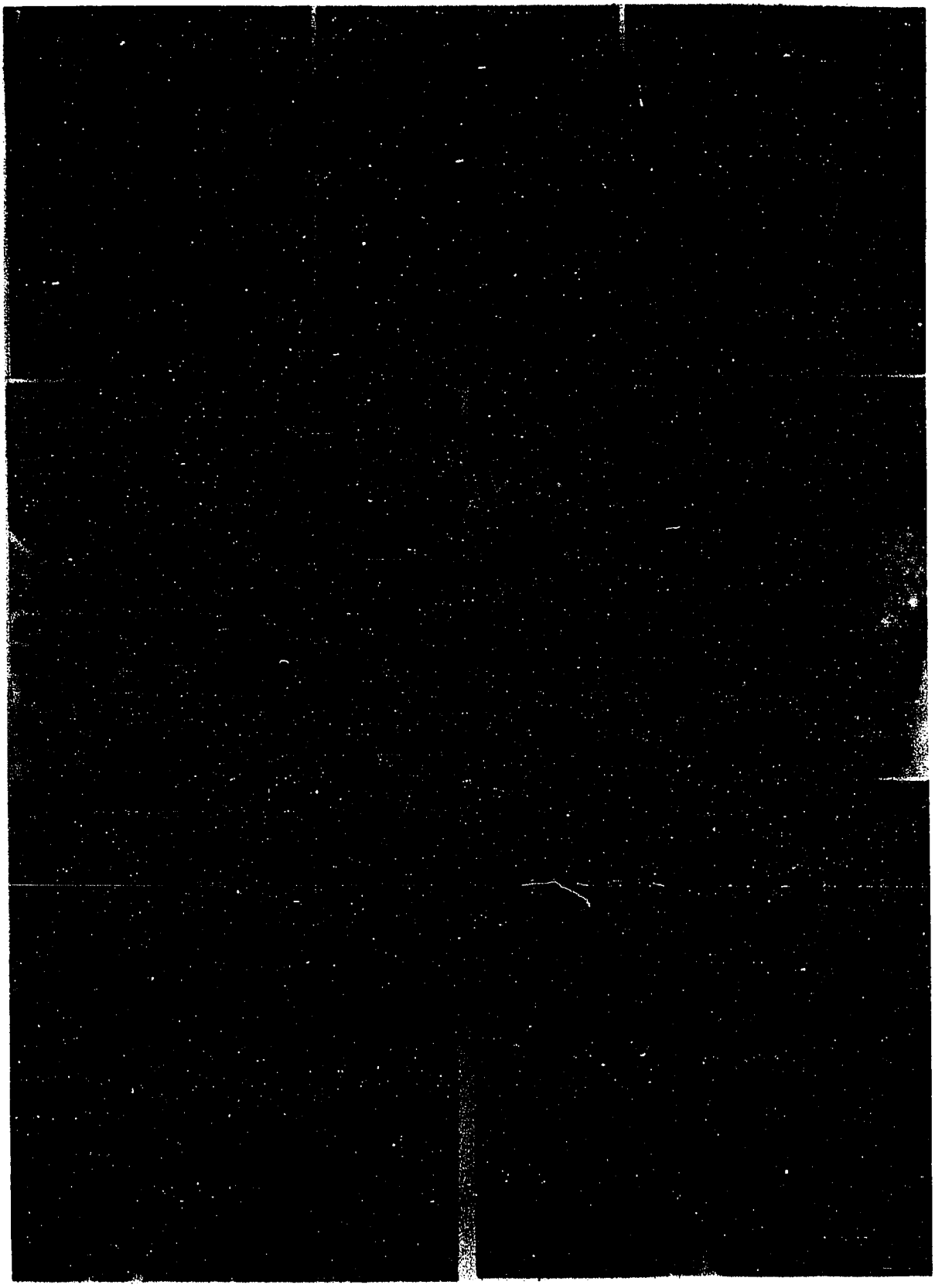

Plate III

Figure 14. An L-fibroblast in prophase showing condensed chromosomes (C), many in close relationship with the nuclear membrane (Nm). Two late telophase fibroblasts occur on the right side of the picture. Portions of the reforming nuclear membrane (Nm) are distinct, as is a large Golgi zone (Gz) facing the equatorial plate. DMP 1317X

Figure 15. The same cells as in Figure 14 showing relatively high accumulations of BP in the Golgi zone (Gz), in the nuclear membrane (Nm) of prophase prior to its breakdown, and in the nuclear membrane (Nm) forming around the swelling karyomeres of telophase. The region of the nucleus surrounded by the nuclear membrane shows no apparent uptake of BP in either prophase or telophase nuclei. UVFM 1317X

Figure 16. A mitotic L-fibroblast in metaphase showing the metaphase plate at right angles to the viewer. No apparent BP uptake occurs in the chromatid pairs (C) lined up on the metaphase plate. UVFM 1317X

Figure 17. Same cells as in Figure 16 showing the masses of the chromatid pairs (C) alined on the metaphase plate. Between and surrounding the chromosomal material is a relatively clear perichromosmal cytoplasm (Cp). DMP 1317X

Figure 18. Same cell as in Figures 16 and 17 at a slightly different focal plane than that in Figure 16. Uptake of BP by the perichromosmal cytoplasm (Cp) in and around the metaphase plate is

visible. UVFM 1317X

Figure 19. An L-cell in anaphase showing the migrating daughter chromosomes (C) and the elongating interzonal spindle (I) with no detectable interzonal spindle fibers for any astral structures. DMP 1317X

Figure 20. Same L-cell as in Figure 19 showing no apparent BP uptake by the anaphase chromosomes (C) or in the general interzonal spindle region (I) except for relatively high BP accumulations in Golgi granules (G) which seem to occur within the interzonal spindle as well as the general peripheral cytoplasm. UVFM 1317X

Figure 21. Same cell as in Figures 19 and 20 at a slightly different focal plane than that in Figure 19. An apparent Golgi granule (G) is seen in the general interzonal spindle region. DMP 1317X

Figure 22. An L-929 fibroblast in late anaphase showing the well separated daughter chromosomes (C) surrounded by clear perichromosomal cytoplasm (Cp). The characteristic cytoplasmic blebbing (B) of anaphase is visible in both daughter halves. DMP 1317X

Figure 23. Same cell as in Figure 22 showing no apparent BP uptake in daughter chromosomes (C) or in the internum of cytoplasmic blebs (B). BP accumulations in the periphromosomal cytoplasm (Cp) are comparable with those in the general cytoplasmic matrix. UVFM 1317X

Figures 24 and 25. An L-fibroblast in late anaphase showing the interzonal spindle region (I) and peripheral Golgi granules (G). DMP 1317X

Figure 26. Same cell as in Figure 24 and 25 showing no demonstrable BP uptake in late anaphase chromosomes (C) and the interzonal spindle region (G), but high apparent accumulations of BP in Golgi granules (G) at the periphery of the spindle. UVFM 1317X

Figure 27. Same cell as in Figures 24, 25 and 26 showing apparent interzonal spindle fibers (If). DMP 1317X

Figure 28. A pair of L-cells in telophase in the upper portion of the photomicrograph and an interphase fibroblast below. The enlarging karyomeric masses (K) are visible, and the equatorial plate (Eq), dividing the daughter halves, is seen as a constriction in the cytoplasmic bridge connecting the two cells. DMP 1317X

Figure 29. Same cells as in Figure 28 showing no demonstrable BP uptake in any portion of the karyomeric aggregate (K). The newly formed portions of the nuclear membrane show apparent BP accumulation (Nm) as does the cytoplasm involved in the equatorial plate (Eq). UVFM 1317X

Figure 30. Same cells as in Figures 28 and 29 at a slightly different focal plane showing portions of a distinct nuclear membrane (Nm) in the process of reconstruction at the surface of the karyomeric aggregate. DMP 1317X

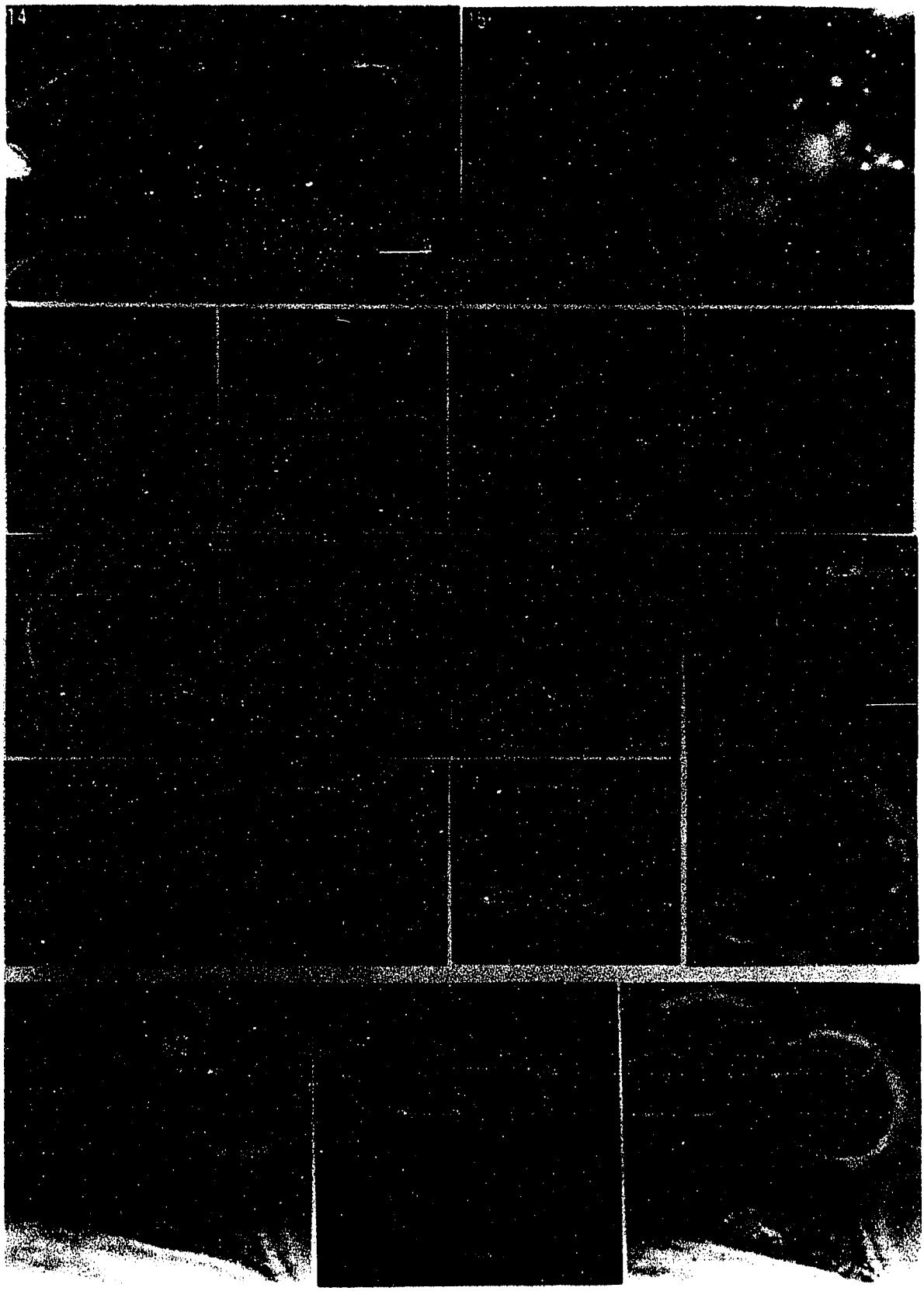
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Plate IV

Figure 31. An L-fibroblast observed on a crystalline bed of pyrene showing portions of the granular component (sg) throughout the cytoplasm. The labeled granules show evidence of fusion. DMP 1317X

Figure 32. Same L-cell as in Figure 31 but viewed at a higher focal plane. Nuclear structures, including compact and vacuolated nucleoli (nc) and heterochromatin, are in focus. The variety in size of the granular component (sg) is apparent. DMP 1317X

Figure 33. Same cell as in Figures 31 and 32 showing no apparent pyrene uptake in any nuclear structure (N) or in the granular component (sg). UVFM 1317X

Figure 34. Portions of two L-cells on a crystalline bed of pyrene showing the variation in size of the granular component (sg), and the heterogeniety of the larger granules. DMP 1317X

Figure 35. The same cells as in Figure 23 at a slightly higher focal plane showing the nuclear structures of these cells, including heterochromatin (Hc) and large nucleoli (nc). DMP 1317X

Figure 36. The same cells as in Figures 34 and 35 showing no apparent uptake of pyrene in any nuclear structure (N) or in the granular component (sg). UVFM 1317X

Figure 37. L-cells, grown on crystalline beds of BP and concomitantly exposed to croton oil, showing relatively high BP uptake in Golgi granules (G), the nuclear membrane (Nm) and the rim of the cytoplasmic bleb (B). No apparent BP uptake is seen in anaphase chromosomes (C), in any structure within the confines of the nuclear membrane

(N) or in the internum of the cytoplasmic blebs. (B). UVFM 1317X

Figure 38. Same cells as in Figure 37 showing the reactions of L-cells simultaneously exposed to BP and croton oil. The fibroblast on the right side of the picture shows a distinct nuclear membrane (Nm), heterochromatin (Hc), nucleoli (nc) and large cytoplasmic blebs (B). The cell on the left is in anaphase and shows the daughter chromosomes (C), interzonal spindle (I) and Golgi granules (G) in the peripheral cytoplasm. DMP 1317X

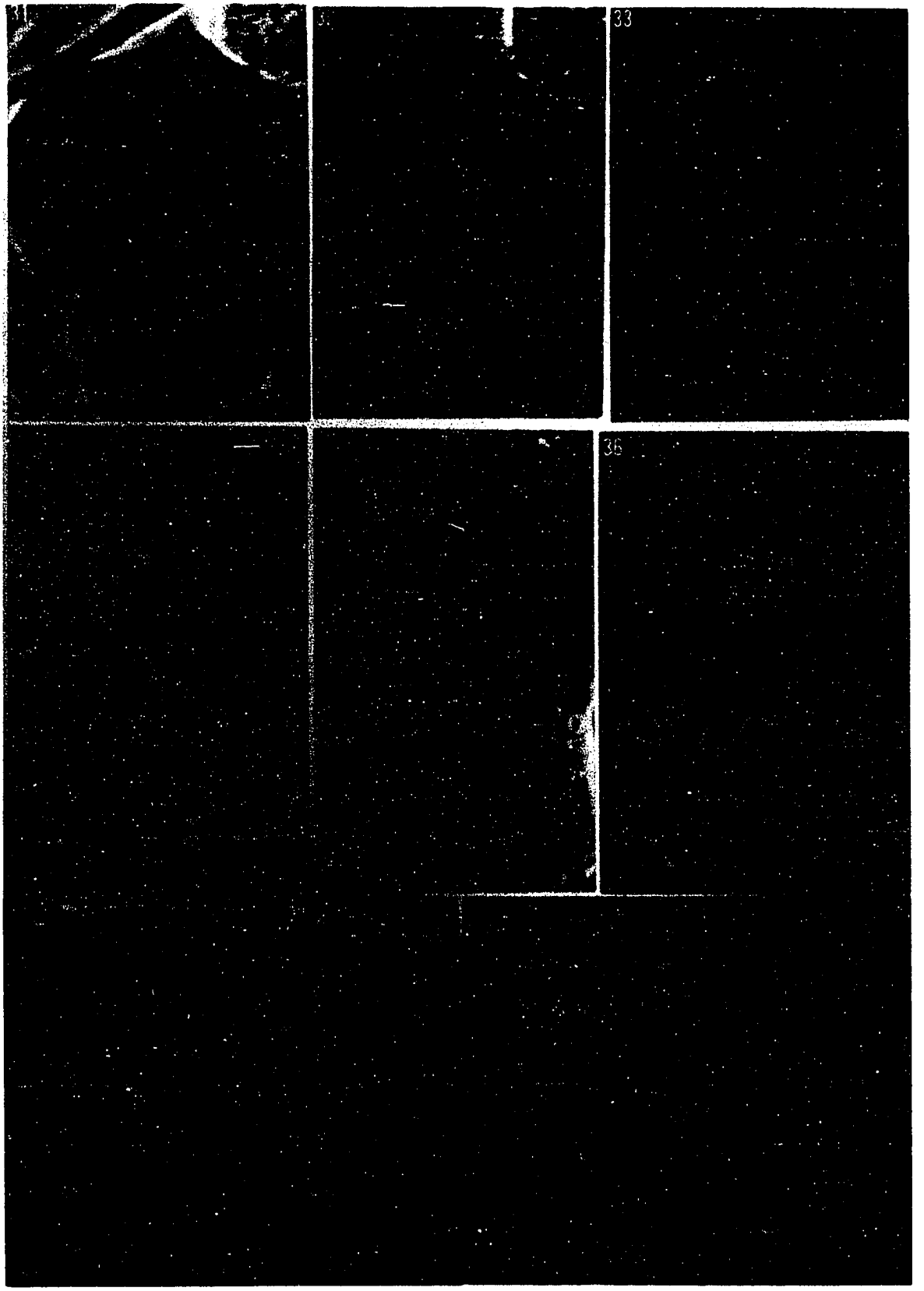
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Plate V

Figure 39. Portions of L-929 fibroblasts fixed with formalin vapors and exposed to BP on crystalline beds. Much of the cytoplasm is filled with small vesicles (V), especially in the case of the cell in the upper middle of the picture. Nuclei possess a distinct nuclear membrane (Nm) and nucleoli (nc). DMP 1317X

Figure 40. Same cells as in Figure 39 showing no demonstrable BP uptake in any nuclear structure (N) or in the cytoplasmic vesicles (V). UVFM 1317X

Figure 41. L-cells fixed in $CaCO_3$-HCHO for one hour after being grown on a crystalline bed of BP in vitro. The cell shows Golgi granules (G), elongated mitochondria (M), some cytoplasmic vacuoles and free vesicles (V) which have been formed from the cell surface during fixation. DMP 1317X

Figure 42. Same cells as in Figure 41 showing relatively high BP uptake by the Golgi granules (G) and no demonstrable uptake by mitochondria (M), the internum of the vesicles (V) or by any nuclear structure (N). UVFM 1317X

Figure 43. A single L-cell fixed one hour in 40% HCHO showing high apparent BP concentrations in Golgi granules (G) and no apparent accumulation in the nucleus (N) or in cytoplasmic vacuoles (V). Uptake by the fine cytoplasmic processes (Fp) is similar to that by the general cytoplasmic matrix. UVFM 1317X

Figure 44. The same cell as in Figure 43 showing the highly refractile nucleus (N), vacuolated cytoplasm (V) and intact fine

cytoplasmic processes (Fp). The cell process on the right side of the cell shows some fibrous material. DMP 1317X

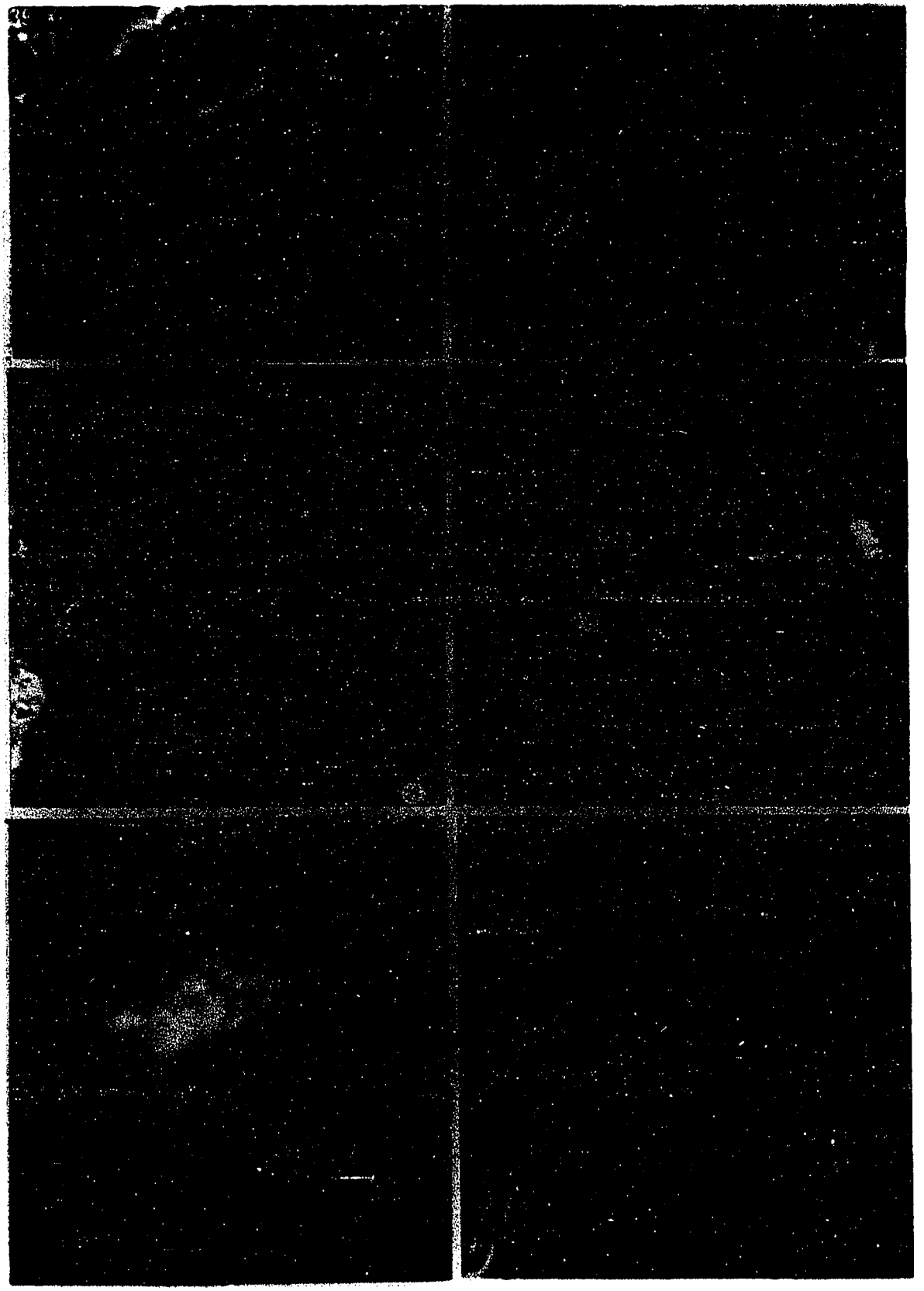

Plate VI

Figure 45. An L-929 fibroblast not grown on BP *in vitro*, fixed with $CaCl_2$-HCHO. The focal plane is low relative to the nucleus (N) so that nuclear structures are indistinct. The hyaline cytoplasmic matrix in which strands of fibrous material (F) are embedded are visible in the broad upper end of the cell. Golgi granules (G), mitochondria (M) and cytoplasmic vacuoles appear discrete. DMP 1317X

Figure 46. The same cell as in Figure 45 showing relatively high concentration of BP in Golgi granules (G) and no apparent CH uptake by mitochondrial (M) or vacuolar structures (Va). The nucleus is interpreted to show no BP uptake, the fluorescence associated with it is resident in the underlying cytoplasm. UVFM 1317X

Figure 47. An L-fibroblast grown on BP and fixed one hour in $CaCl_2$-HCHO showing BP uptake in relatively high concentrations by the Golgi zone (Gz) and the cytoplasmic tongue in the nucleus (Ic). Mitochondria (M), cytoplasmic vacuoles (Va) and all nuclear structures display no apparent accumulations of BP. UVFM 1317X

Figure 48. The same cell as in Figure 47 showing rod-shaped mitochondria (M), irregular-shaped vacuolar spaces (Va), a Golgi zone (Gz) filled with strands and vesicles which are seemingly continuous with a tongue of cytoplasm (Ic) extending into the nucleus. Within the nucleus, the nuclear margin blends internally with the undifferentiated non-nucleolar karyoplasm. No distinct chromatin structure is visible. DMP 1317X

Figure 49. The same cell as in Figures 47 and 48 at a slightly

different focal plane to show more detail of the cytoplasmic tongue in the nucleus (Ic). DMP 1317X

Figure 50. A mitotic cell grown on BP and fixed one hour in $CaCl_2$. Chromatin material shows no apparent BP uptake (C). UVFM 1317X

Figure 51. The same metaphase cells as in Figure 50. The indistinct structure of the chromosomes (C) on their metaphase plate is visible. DMP 1317X

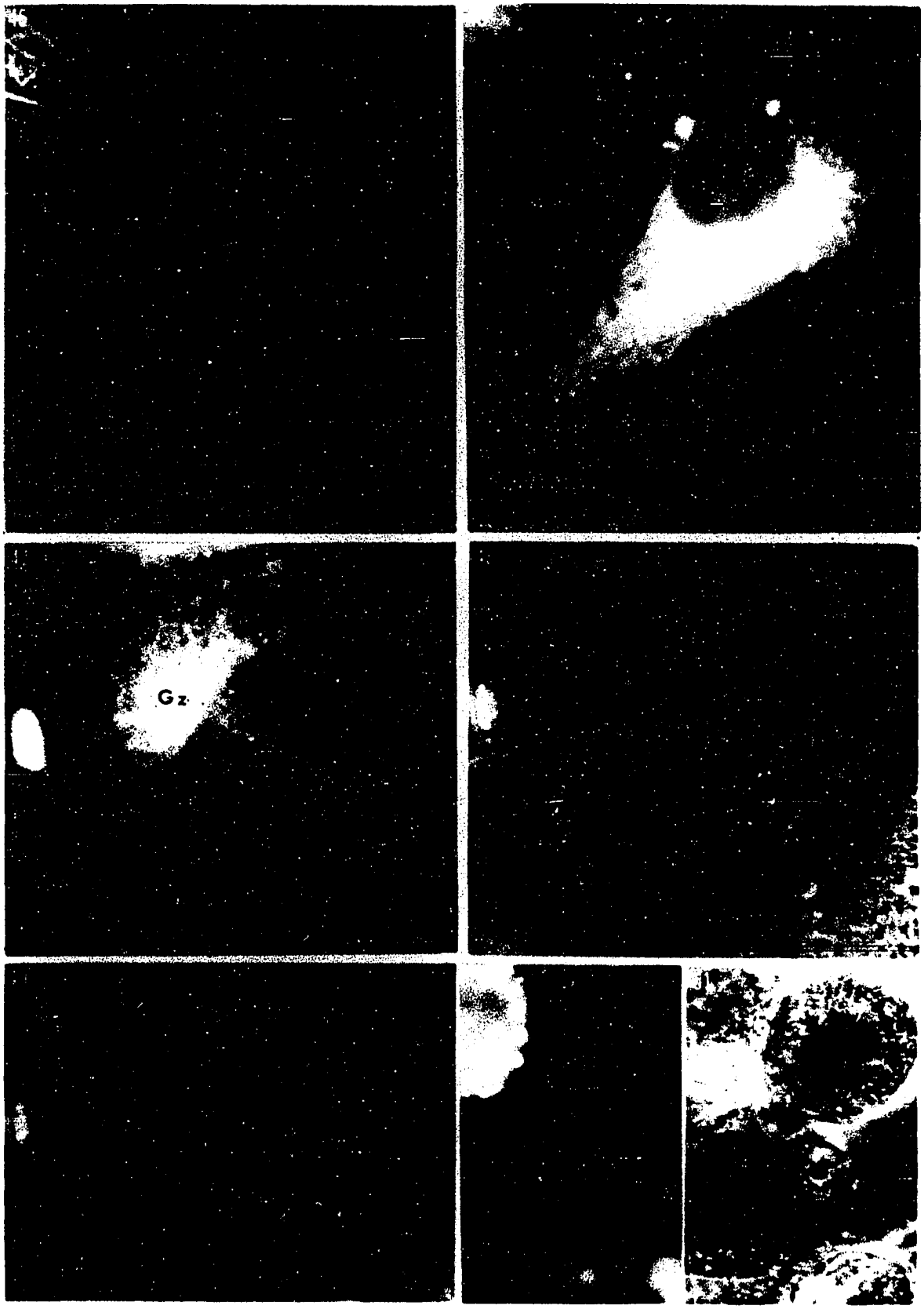
Gz

Plate VII

Figure 52. A fibroblast fixed with Lillie's HCHO for five minutes and treated with Berg's caffeine-BP solution. A few vacuoles (V) are visible in the central cytoplasmic region and Golgi granules (G) in the intermediate cytoplasm. A distinct nuclear membrane and nucleoli (nc) are seen in the cell nucleus, but the chromatin (Hc) is poorly resolved. DMP 1317X

Figure 53. The same cell as in Figure 52 showing high levels of BP uptake in the Golgi granules (G) and no demonstrable CH in the cytoplasmic vacuoles (V). All nuclear structures (N) display a lack of BP uptake. UVFM 1317X

Figure 54. The same cell as in Figures 52 and 53 observed at a slightly lower focal plane to show the hyaline cytoplasm of the velamentous process in the upper part of the picture and the interwoven fibrous material (F) within that clear cytoplasm. DMP 1317X

Figure 55. A group of L-929 fibroblasts grown on a bed of BP in vitro and fixed for one hour in Lillie's HCHO. Golgi granules (G) and mitochondria (M) are preserved in the cytoplasm, and lipid droplets (L) are seen in the left hand cell. All the cell nuclei show distinct nuclear margins and nucleoli but indistinct chromatin structure. DMP 1317X

Figure 56. The same group of cells as in Figure 55 showing accumulations of BP in lipid droplets (L) and Golgi granules (G) which are apparently greater than in the gerneral cytoplasmic matrix.

Neither mitochondria (M) or any nuclear structure (N) show any BP accumulation of BP. UVFM 1317X

Figure 57. A group of L-929 fibroblasts seen after fixation in Lillie's HCHO for 60 hours. Relatively high BP uptakes are seen in the Golgi granules (G), while no demonstrable BP accumulation can be seen in mitochondria (M) or in any structure associated with the nucleus (N). UVFM 1317X

Figure 58. The same group of cells as in Figure 58 showing dark mitochondria (M) and Golgi granules (G) in the cytoplasm, and dark nuclear margins and nucleoli (nc) embedded in a karyolymph with distinct chromatin structures. DMP 1317X

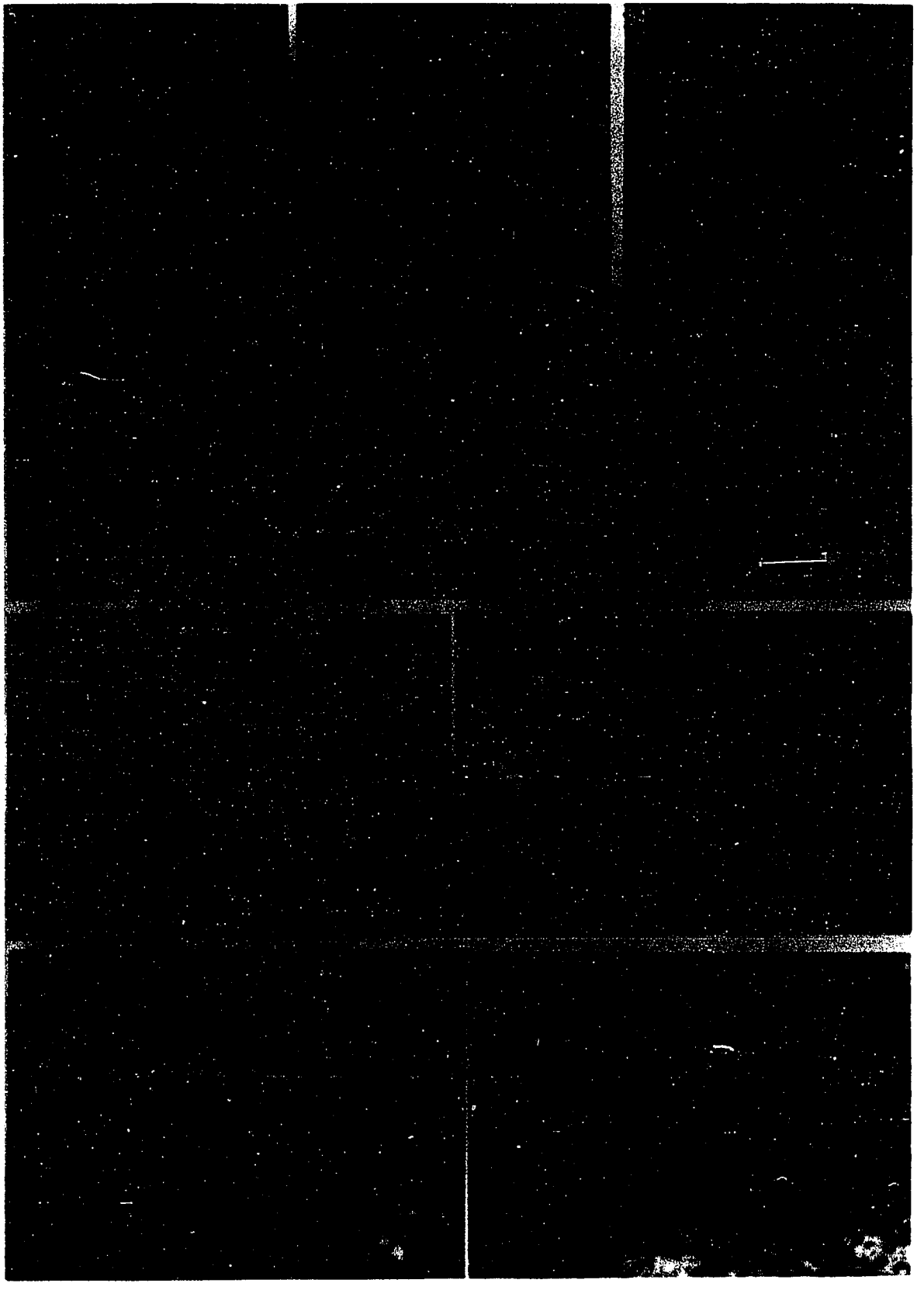

Plate VIII

Figure 59. Several L-fibroblasts fixed one hour in $CaCO_3$-HCHO after being grown on pyrene in vitro. Variably-sized irregular structures occur in the cytoplasm which, peripherally, is clear. A few vacuolar structures are visible (B). Vaguely discernible chromatin (Hc) is seen within the nucleus. DMP 1317X

Figure 60. The same cells as in Figure 59 showing no demonstrable pyrene uptake in vacuolar structures (V) or in any nuclear structure (N). UVFM 1317X

Figure 61. A fibroblast fixed one hour in $CaCl_2$-HCHO and exposed to crystalline beds of Pyrene on clean glass slides. The same cytomorphologic features as were seen in Figure 59 are apparent in this cell. DMP 1317X

Figure 62. The same fibroblast as in Figure 62 showing lack of pyrene accumulation in the nucleus (N). This photomicrograph was obtained after a long exposure time (approximately four minutes). UVFM 1317X

Figure 63. A spindle-shaped fibroblast fixed one hour in $CaCl_2$-HCHO, dehydrated with EtOH and cleared in toluene. The whole cell is generally highly refractile. The cytoplasm is filled with a coarse precipitate and some vacuolar structures (V). Nucleoli are refractile (nc), and the chromatin structure (Hc) is more distinct that in cells not post-treated. A loss of continuity of the nuclear membrane is also apparent. DMP 1317X

Figure 64. The same cell as in Figure 63 showing relatively

lower BP accumulations in cytoplasmic vacuoles (V). Demonstrable uptake of the CH is seen in nucleoli (nc) and in chromatin structures of the nucleus (N). UVFM 1317X

Figure 65. Portions of two cells fixed in $CaCl_2$-HCHO and dehydrated in graded EtOH followed by clearing in toluene. The coarsely precipitated cytoplasm contains irregular vacuolar structures. The nucleoli (nc) are highly refractile and distinct nuclear chromatin is apparent (Hc). The nuclear membrane shows loss of structural integrity over large portions of its course around the nuclear margin. DMP 1317X

Figure 66. The same cells as in Figure 65 showing an indistinctly differentiated pattern of cytoplasmic BP and distinct accumulation of the CH by nucleoli (nc). No apparent uptake of BP by chromatin structures is seen (N). UVFM 1317X

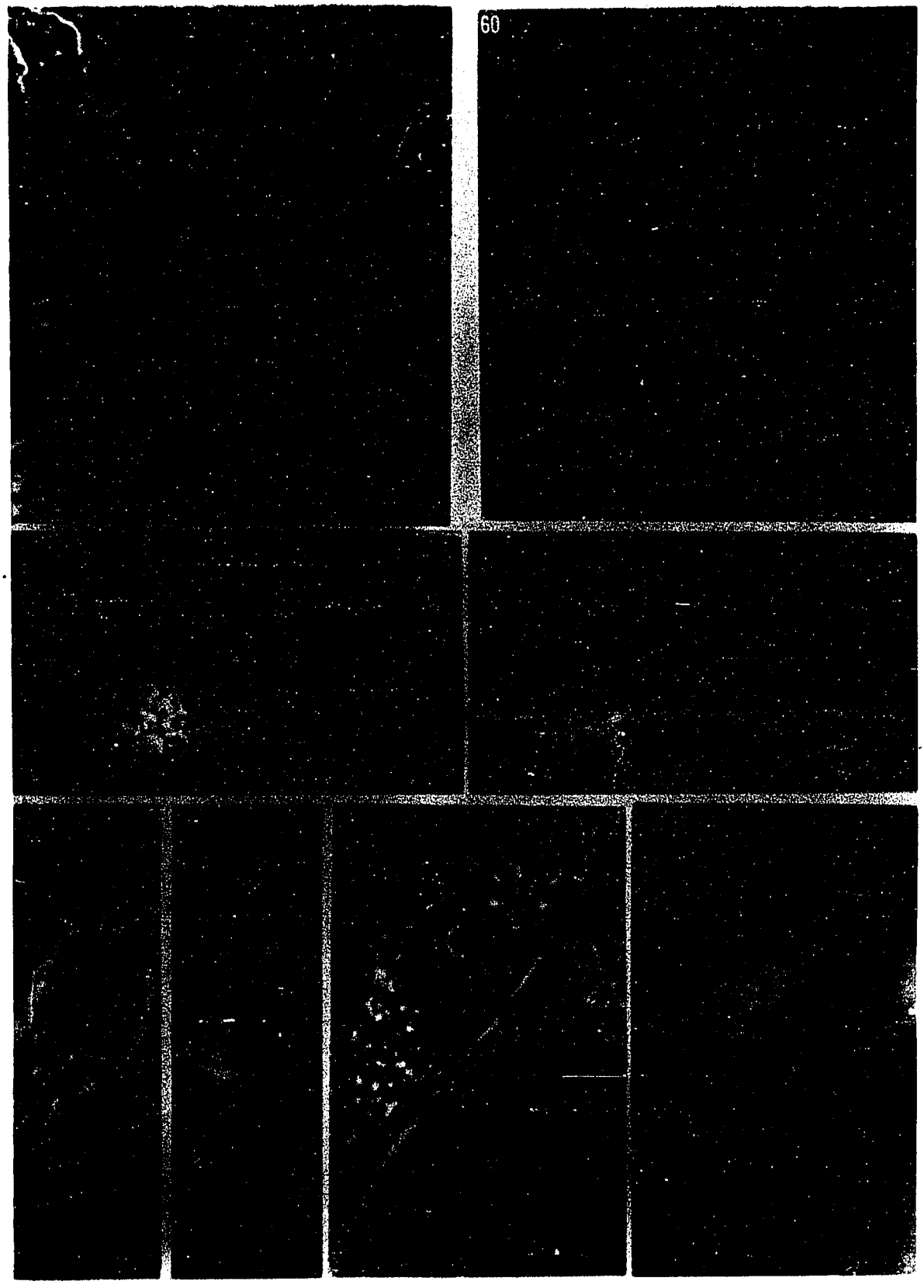
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Plate IX

Figure 67. An L-929 fibroblast frozen in liquid nitrogen by immersion of the culture vessel, containing the living cell, directly into the supercooled liquid. Post-freezing treatment included fixation in Lillie's HCHO and exposure to crystalline BP. BP accumulation is seen in Golgi granules (G). No demonstrable BP concentrations are apparent in cytoplasmic vacuoles (V), mitochondria (M) or in any nuclear structure (N). UVFM 1317X

Figure 68. The same cell as in Figure 67 showing Golgi granules, irregular vacuolar structures (V) and mitochondria, (M) in the cytoplasm. DMP 1317X

Figure 69. An L-929 fibroblast rapidly frozen in liquid nitrogen-chilled isopentane vapors followed by fixation in Lillie's HCHO. Relatively high levles of BP accumulation are present in Golgi granules (G), while no discernible uptake occurs in mitochondria (M) or in any nuclear structure (N). UVFM 1317X

Figure 70. The same fibroblast as in Figure 69 showing Golgi granules (G), mitochondria (M) and a general lack of discrete chromatin structure in the nucleus (N). DMP 1317X

Figure 71. An L-fibroblast frozen rapidly by direct immersion into liquid nitrogen followed by fixation for one hour in Lillie's HCHO. The fibrous material of the cytoplasm (F) seems exaggerated with the formation of vacuolar structures within the confines of its network. Golgi granules are present (G). The nucleus is filled with amorphous clumps of unidentifiable material (N). DMP 1317X

Figure 72. The same cell as in Figure 71 showing demonstrable BP uptake in fibrous material (F) and in Golgi granules (G), but no demonstrable uptake in any nuclear structure (N). UVFM 1317X

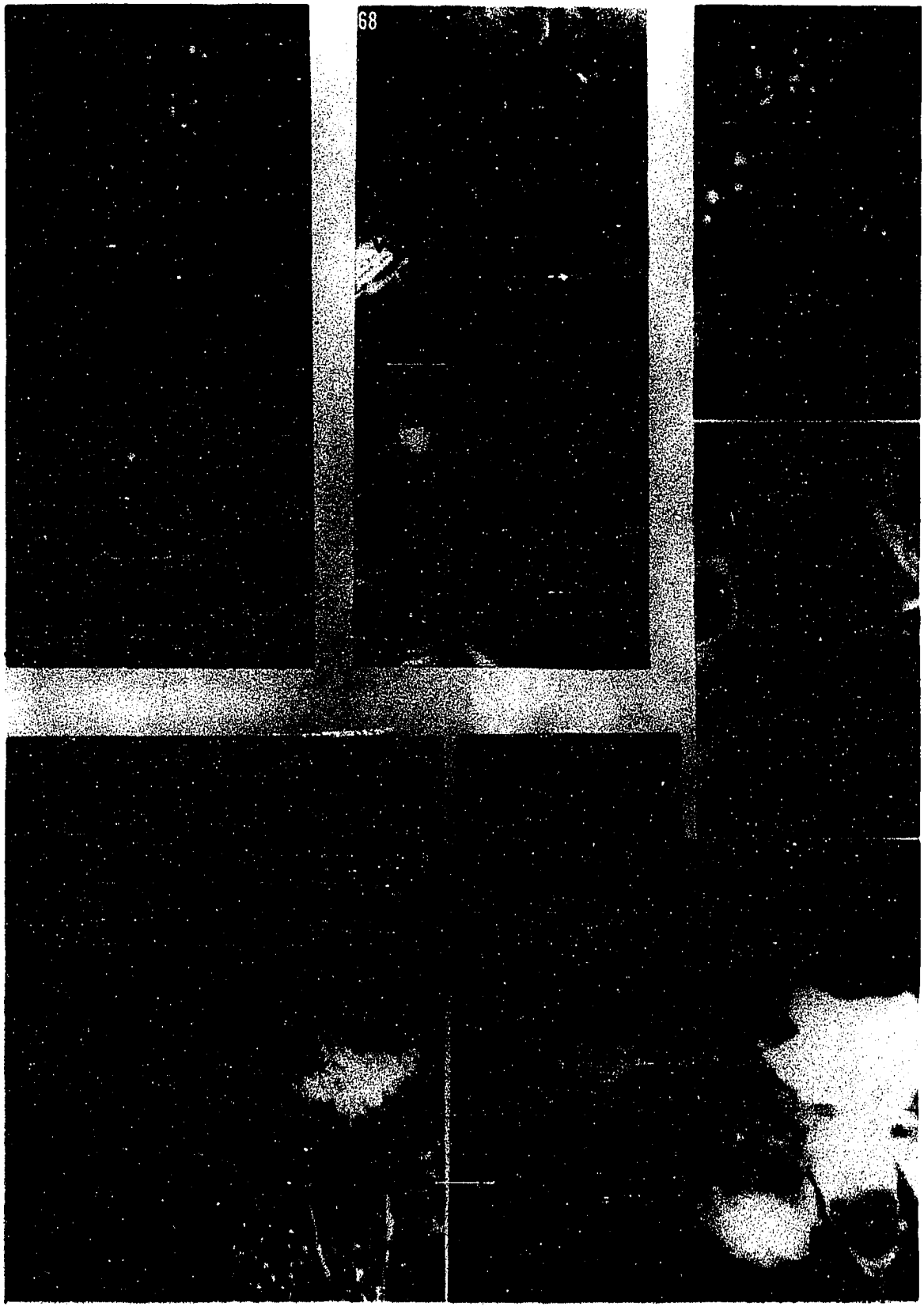
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Plate X

Figure 73. An L-929 fibroblast fixed for one hour in fresh complete Zenker's fluid. Golgi granules (G) and vacuolar structures (V) are the only identifiable features of the precipitated cytoplasm. The nucleus (N) is circumscribed by a discrete nuclear membrane, but the karyoplasm is composed of irregular masses of precipitated material. DMP 1317X

Figure 74. The same cell as in Figure 73 showing relatively high BP uptake in the Golgi granules (G) and no demonstrable uptake of the CH by cytoplasmic vacuoles (V) or by any structure associated with the nucleus (N). UVFM 1317X

Figure 75. A spindle-shaped fibroblast fixed in fresh complete Zenker's solution showing some Golgi granules in an otherwise highly refractile cytoplasm. The nucleus is also highly refractile (N), but a discrete nuclear membrane is seen. DMP 1317X

Figure 76. The same cell as in Figure 75 showing relatively high concentrations of BP situated in the Golgi granules (G) but no apparent uptake by any nuclear structure (N). UVFM 1317X

Figure 77. An interphase and a metaphase fibroblast fixed in complete Zenker's fluid followed by post-treatment with Lugol's solution and thiosulfate. A discrete nucleolus (nc) and finely precipitated chromatin material (Hc) are seen in the interphase nucleus. The metaphase chromosomes are structurally indiscrete (C) and are only more/less outlined by the intervening perichromosomal cytoplasm. DMP 1317X

Figure 78. Two L-929 fibroblasts, one in interphase and the other in telophase showing no discernible BP uptake in any structure associated with the interphase nucleus (N) or in the daughter karyomeres (K). UVFM 1317X

Figure 79. The same cells as in Figure 77 showing no demonstrable BP uptake in any structure associated with the interphase nucleus (N) or in the chromosomes on the metaphase plate (C). UVFM 1317X

Figure 80. The same cells as in Figure 78. The interphase nucleus contains discernible nucleoli and fine chromatin material. The daughter karyomeres are without apparent structural differentiation (K). Interzonal spindle fibers are distinct (IF) as is the area of the equatorial plate (Eq). DMP 1317X

Figure 81. An L-929 fibroblast in relatively late anaphase fixed in fresh, complete Zenker's fluid followed by post-treatment to remove all mercury. The daughter chromosomes (C) are outlined by the perichromosomal cytoplasm. Interzonal spindle fibers lie between the separating chromosomal masses, embedded in a relatively clear interzonal spindle region. DMP 1317X

Figure 82. The same anaphase cell as in Figure 81 showing no apparent uptake of BP by the daughter chromosomes (C). The general interzonal spindle region (I) showed a BP uptake comparable with that by the general cytoplasmic matrix. UVFM 1317X

Figure 83. The same anaphase cell as in Figure 81 and 82 showing the perichromosomal cytoplasm in more detail. DMP 1317X

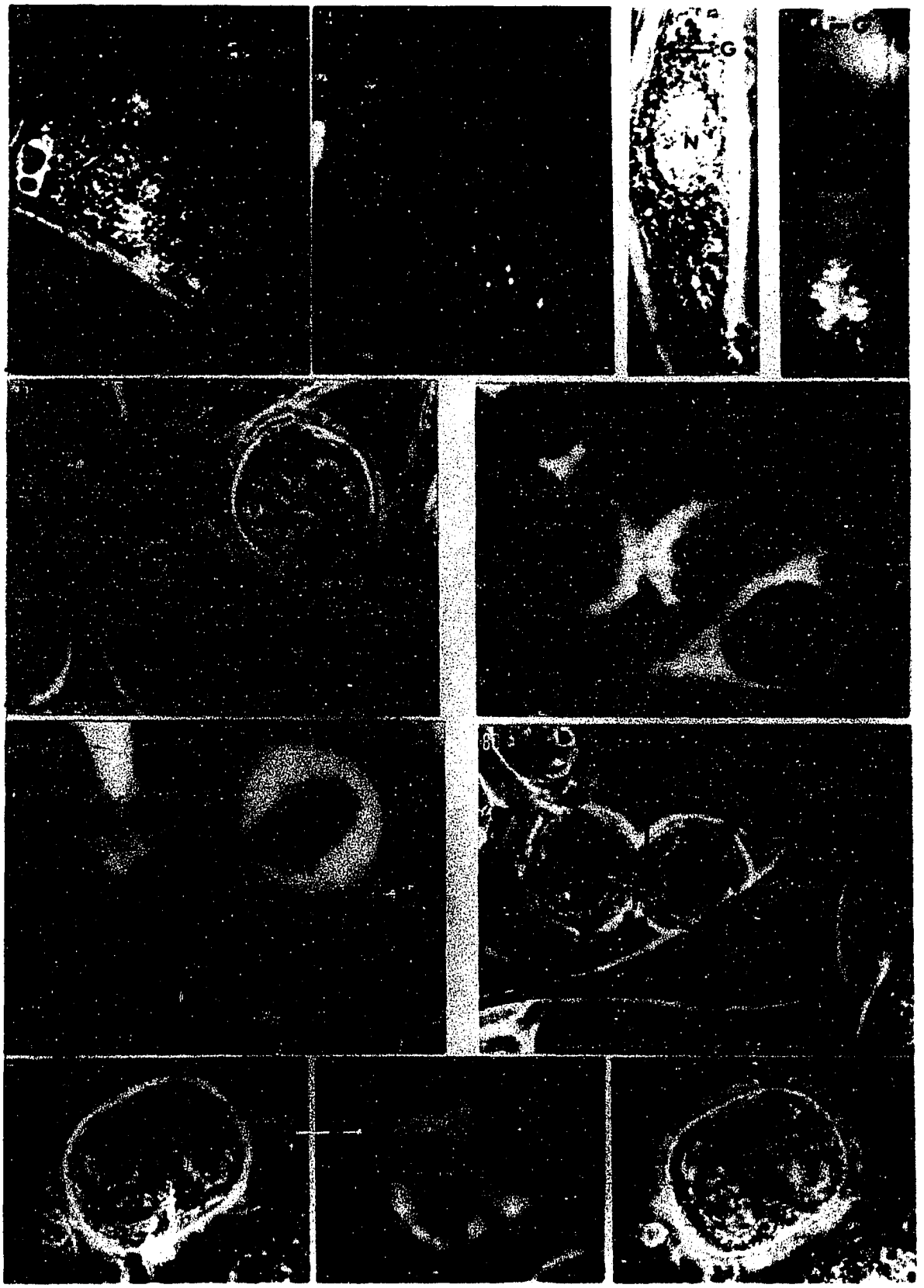
G
N
G

Plate XI

Figure 84. An L-929 fibroblast fixed one hour in aged, complete Zenker's fluid followed by post-treatment with Lugol's solution and thiosulfate. The focal plane of the picture is lower than most of the nucleus (N), which, nevertheless, is seen to be lobulated. A coarse fibrous material (F) is discernible in the nearly clear cytoplasmic matrix. DMP 1317X

Figure 85. The same cell as in Figure 84 showing apparent lack of BP uptake by any nuclear structure (N). The CH-fluorescence between the secondary nuclear lobule and the main body of the nucleus is due to cytoplasm, with its pronounced BP content, investing the nuclear isthmus. The fibrous material (F) in the cytoplasm shows a BP uptake comparable with that of the cytoplasmic matrix. UVFM 1317X

Figure 86. An L-929 fibroblast fixed in aged Zenker's fluid but not post-treated except for exposure to crystalline beds of BP on clean glass slides. Relatively higher accumulations of BP are visibly localized in Golgi granules (G). No demonstrable BP uptake occurs in any nuclear structure (N). UVFM 1317X

Figure 87. The same cell as in Figure 86 showing cytoplasmic Golgi granules (G). Within the nucleus, the nucleoli (nc) and fine chromatin is visible. The nuclear membrane is absent over approximately one half of the nuclear circumference. DMP 1317X

Figure 88. A group of fibroblasts fixed with Zenker's stock but not post-treated for removal of mercury. In the central cell of the group, Golgi structures are apparent (G), while the nucleus

contains discernible nucleoli (nc), chromatin and a distinct nuclear membrane. DMP 1317X

Figure 89. The same group of cells as in Figure 88 showing relatively higher levels of BP uptake in the Golgi structures (G) and no demonstrable BP uptake in any nuclear structure (N). UVFM 1317X

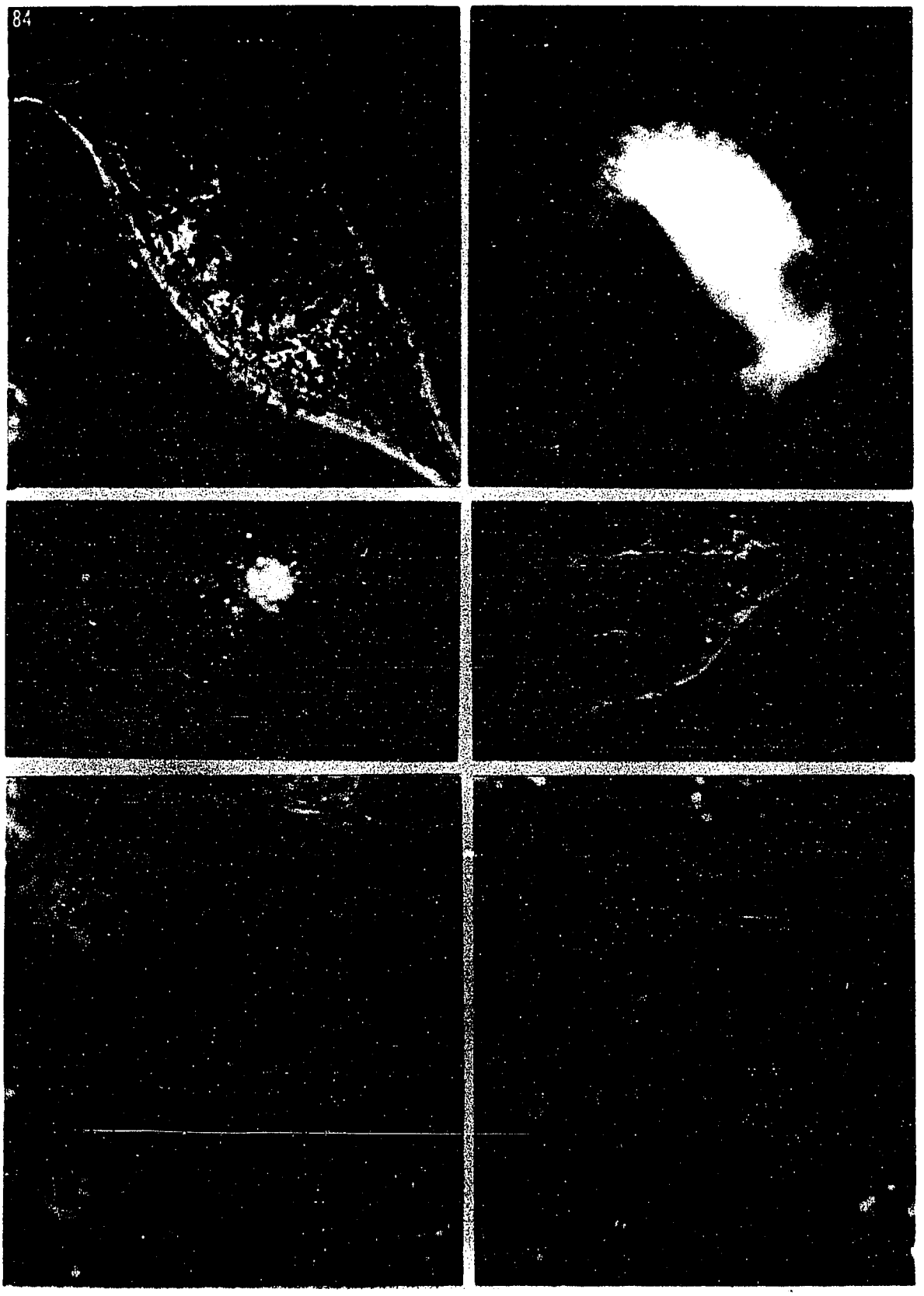
84

Plate XII

Figure 90. A group of L-cells fixed in Zenker's stock with no post-fixation treatment. Mitochondria (M) and Golgi granules (G) are seen in the cytoplasm, while in the nucleus (N), nucleoli, fine chromatin and a distinct nuclear membrane are preserved. DMP 1317X

Figure 91. The same group of cells as in Figure 90 showing relatively higher BP concentrations in the Golgi granules (G) and no apparent BP accumulation by mitochondria (M) or any nuclear structure. (N). UVFM 1317X

Figure 92. Portions of several L-cells fixed in Zenker's stock solution followed by sequential treatment with Lugol's iodine solution and thiosulfate. No demonstrable BP uptake is demonstrable in cytoplasmic vacuoles (V) or in any nuclear structure (N). UVFM 1317X

Figure 93. Portions of the same cells as in Figure 92 showing cytoplasmic vacuoles and nuclear structures, including nucleoli (nc), chromatin and a nuclear membrane. DMP 1317X

Figure 94. A single highly refractile fibroblast fixed in acidified potassium dichromate for one hour. A discernible nuclear membrane (Nm) is seen surrounding a highly refractile nucleus. DMP 1317X

Figure 95. The same cell as in Figure 94 showing relatively high BP uptake in lipid droplets (L), and no apparent BP accumulation in any nuclear structure (N). The CH-fluorescence in the middle of the nucleus is due to overlying cytoplasm. UVFM 1317X

Figure 96. The same cell as in Figures 94 and 95, seen at a focal plane where lipid droplets (L) are distinct and the nucleus (N) visibly filled with a coarse chromatin. DMP 1317X

Figure 97. The distribution of BP in the L-cell remnant after fixation in 5% HOAc for one hour showing relatively higher accumulations of BP in frank lipid droplets (L). The nuclear region (N), which is without discernible material, also shows no localization of the CH. UVFM 1317X

Figure 98. The same cellular remnants as in Figure 97. The region formerly occupied by the nucleus (N) is indicated. The cytoplasm has been reduced to a mass of material including frank lipid droplets (L). DMP 1317X

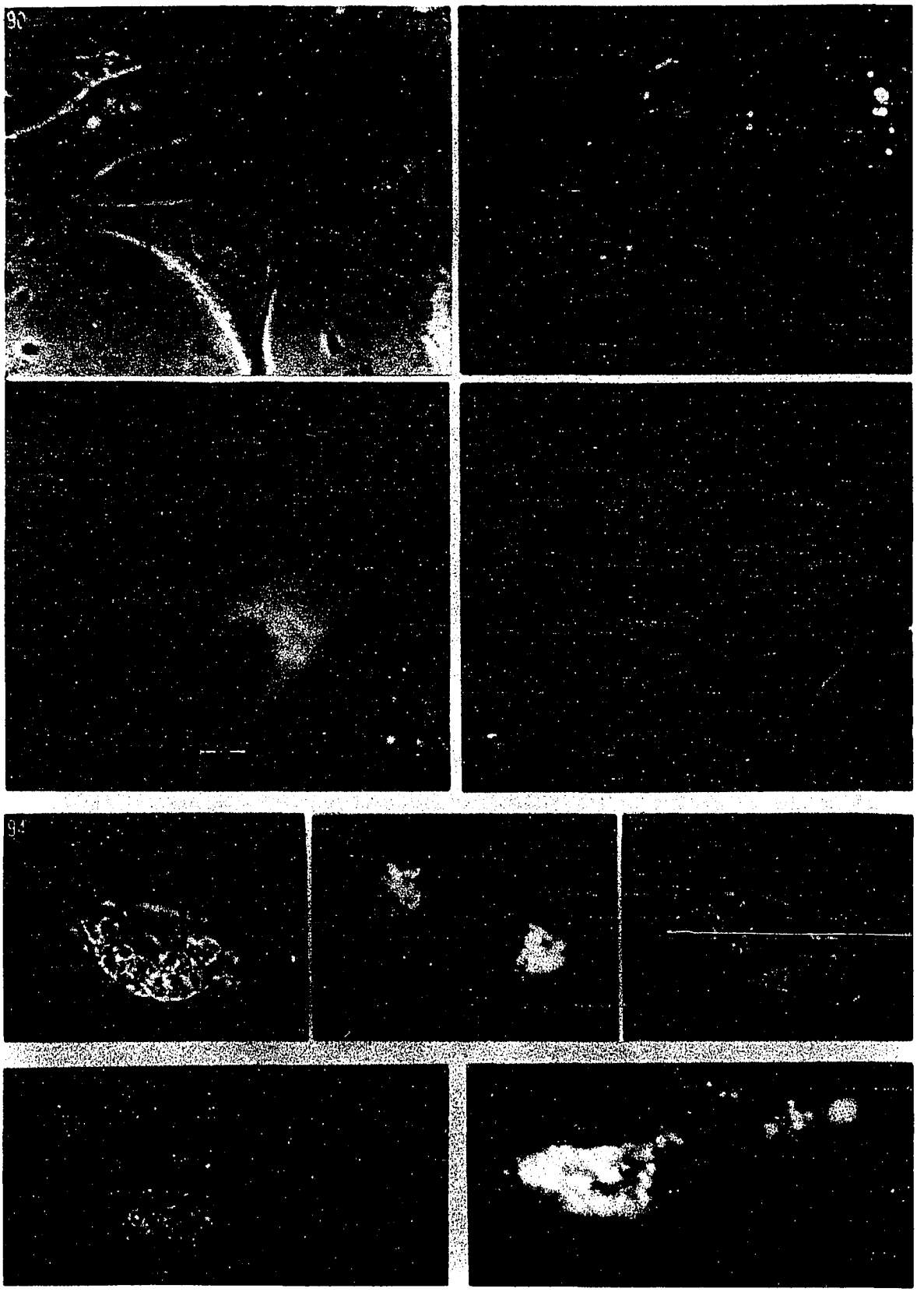

Plate XIII

Figure 99. A large L-fibroblast fixed one hour in a dichromate solution. The cytoplasm is filled with vacuoles (V), and the nucleus (N) is filled with amorphous masses of apparently unstructured material. DMP 1317X

Figure 100. The same cell as in Figure 99 showing no demonstrable BP accumulation in vacuolar structures (V) or in any nuclear structure (N). The over- or underlying cytoplasm with its BP accumulation accounts for the CH-fluorescence visible over the nuclear region. UVFM 1317X

Figure 101. A group of Zenker's fluid-fixed L-929 fibroblasts exposed to crystals of pyrene. The pyrene is seen to be rather unlocalized in the cytoplasm. In general no area of pyrene-fluorescence can be correlated with specific intranuclear structures (N), thus it is interpreted that no intranuclear pyrene uptake occurs. UVFM 1317X

Figure 102. The same group of cells as in Figure 101 showing distinct nuclear structures such as the nuclear membrane, nucleoli (nc) and chromatin. DMP 1317X

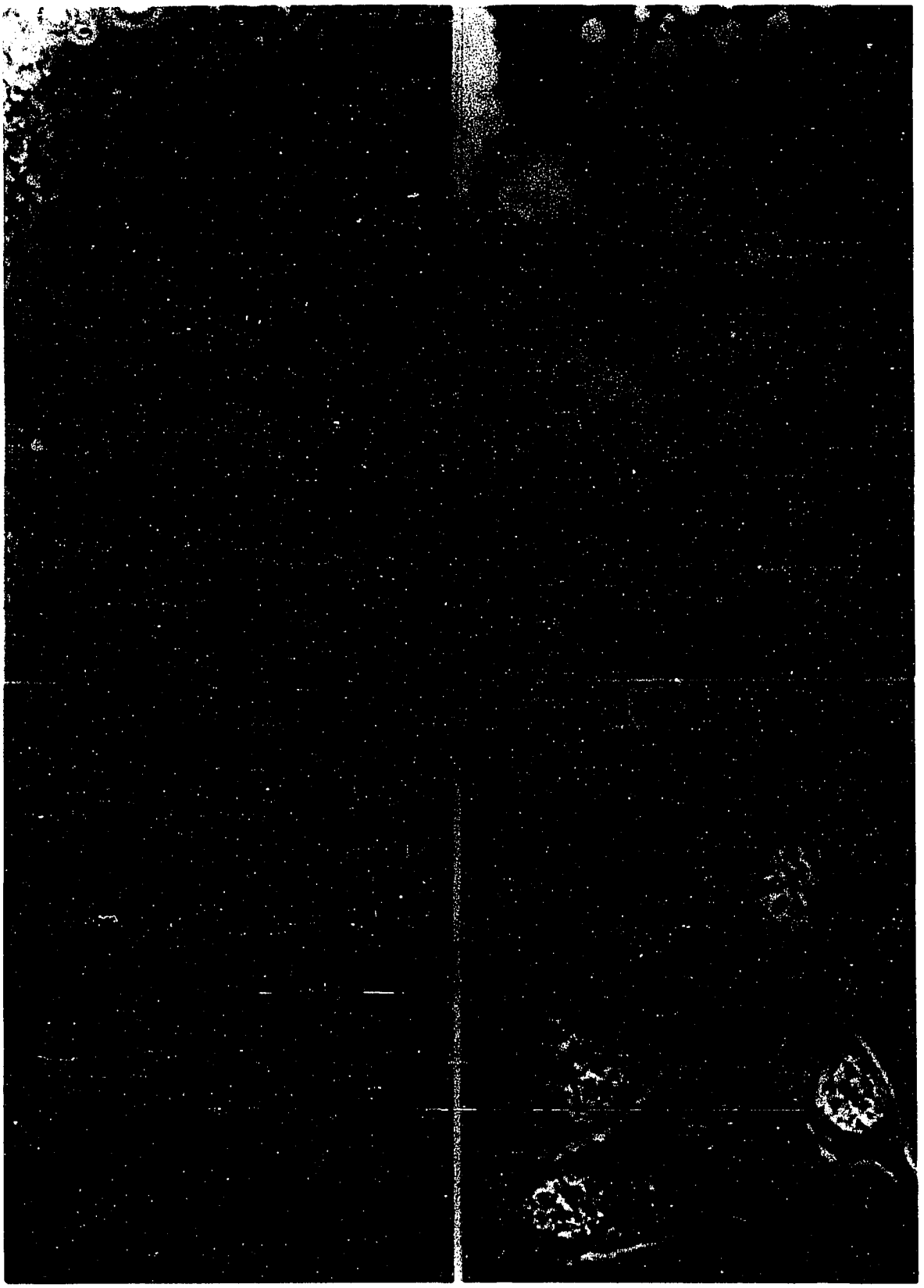

Plate XIV

Figure 103. Portions of several L-cells fixed with $HgCl_2$. Relatively higher accumulations of BP are seen in frank lipid droplets (D), but no demonstrable BP accumulation is apparent in cytoplasmic vacuoles (V) or any nuclear structure (N). UVFM 1317X

Figure 104. The same cells as in Figure 103. The cytoplasm is filled with a coarse precipitate, vacuolar structures (V) and frank lipid droplets (L). The nucleus is surrounded with a distinct but often broken nuclear membrane (Nm) and contains nucleolar structures and a variably coarse chromatin. DMP 1317X

Figure 105. A group of fibroblasts fixed in $HgCl_2$ and post-treated for removal of mercury with Lugol's solution followed by a thiosulfate solution. The highest discernible BP accumulation in the cytoplasm occurs in the lipid droplets (L). No apparent BP accumulation occurs in any nuclear structure (N). UVFM 1317X

Figure 106. The same cells as in Figure 105 showing the coarsely precipitated cytoplasm with occasional lipid droplets (L) and nuclei with coarse chromatin, highly refractile nucleoli (nc) and discrete nuclear membranes (Nm). DMP 1317X

Figure 107. An L-929 fibroblast fixed in $HgCl_2$ and post-treated for removal of mercury showing the coarse cytoplasmic precipitate with large irregular vacuolar spaces (V). Fine cytoplasmic processes are intact (Fp). The cell has been exposed to crystals of pyrene previously prepared on clean glass slides. DMP 1317X

Figure 108. The same fibroblast as in Figure 107 showing an

indistinct cytoplasmic localization of the pyrene. Both the cytoplasmic vacuoles (V) and the nucleus in general (N) show no apparent accumulation of the non-CH. UVFM 1317X

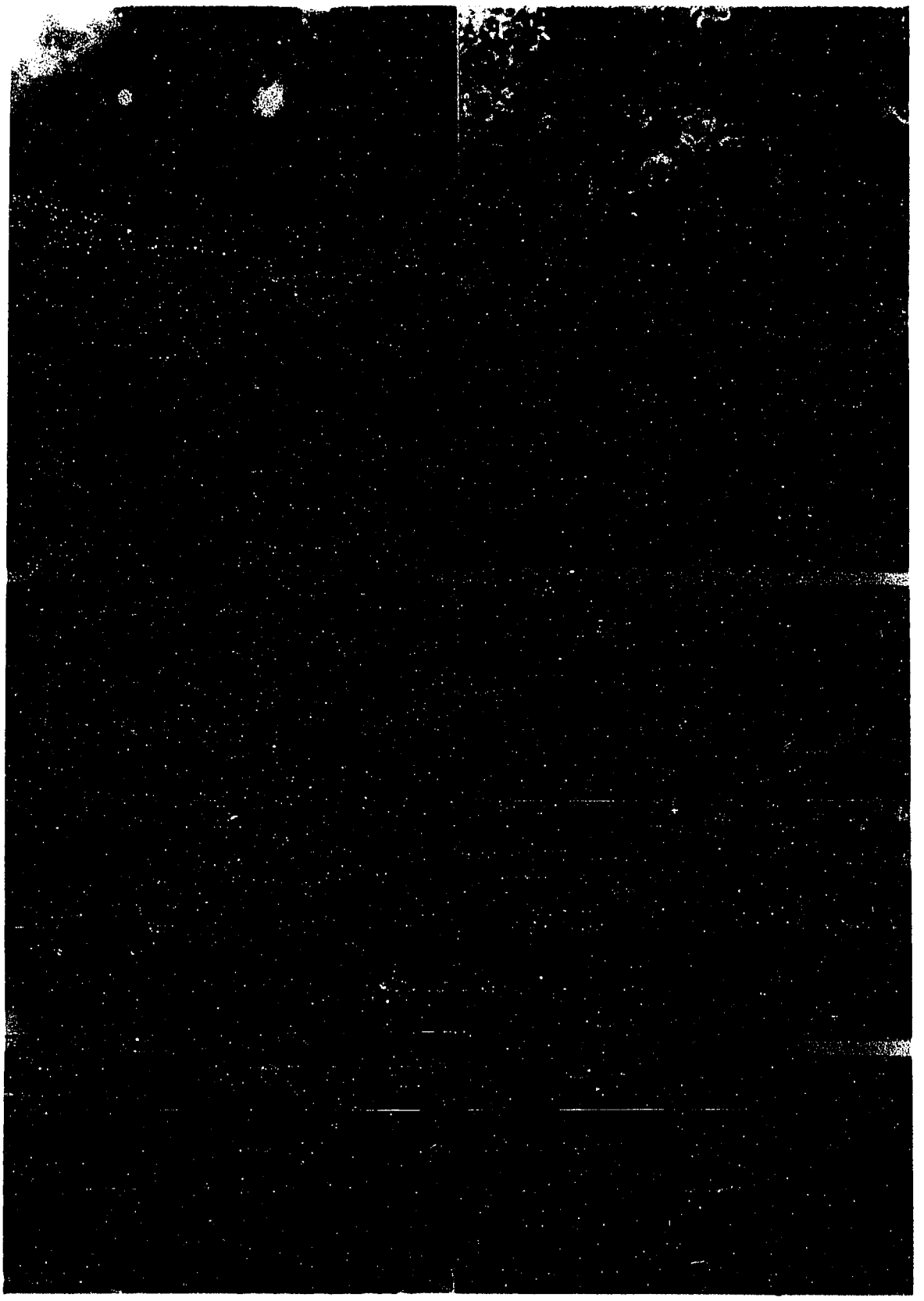

Plate XV

Figure 109. Portions of two L-929 fibroblasts fixed for one hour in 95% EtOH showing a coarsely precipitated cytoplasm and a few cytoplasmic vacuoles (V) and nuclei, containing highly refractile nucleoli (nc). DMP 1317X

Figure 110. The same cells as in Figure 109 showing relatively indiscrete localization of BP in the cytoplasm. Cytoplasmic vacuoles (V) show relatively lower levels of apparent BP uptake than the general cytoplasmic matrix. The nucleoli in both cells show demonstrable accumulation of the CH (nc). UVFM 1317X

Figure 111. The same cells as in Figure 109 and 110 at a lower focal plane. DMP 1317X

Figure 112. Portions of a pair of L-fibroblasts fixed with 95% EtOH. Cytoplasmic vacuoles (V) appear within the coarse protoplasmic precipitate. The nucleus is partially surrounded by a discontinuous nuclear membrane (Nm). Embedded in the fine karyoplasmic precipitate are several nucleoli (nc). DMP 1317X

Figure 113. The same cells as in Figure 112 showing a barely perceptible differential localization of BP in the cytoplasm. Cytoplasmic vacuoles (V) show no apparent uptake of BP. The general karyoplasmic region of the cell nucleus showed no apparent BP uptake except for a demonstrable accumulation in nucleoli (nc). UVFM 1317X

Figure 114. Portions of two L-fibroblasts fixed for only 10 minutes in 95% EtOH. The general nucleocytoplasmic ratio is apparently high. Within the cell nuclei, nucleoli (nc) are distinct, but chromatin

is poorly resolved. DMP 1317X

Figure 115. The same cells as in Figure 115 showing no apparent BP uptake in the cell nuclei except for a possible uptake by nucleolar structures (nc). UVFM 1317X

Figure 116. The same cells as in Figures 114 and 115 showing a slightly different focal plane. DMP 1317X

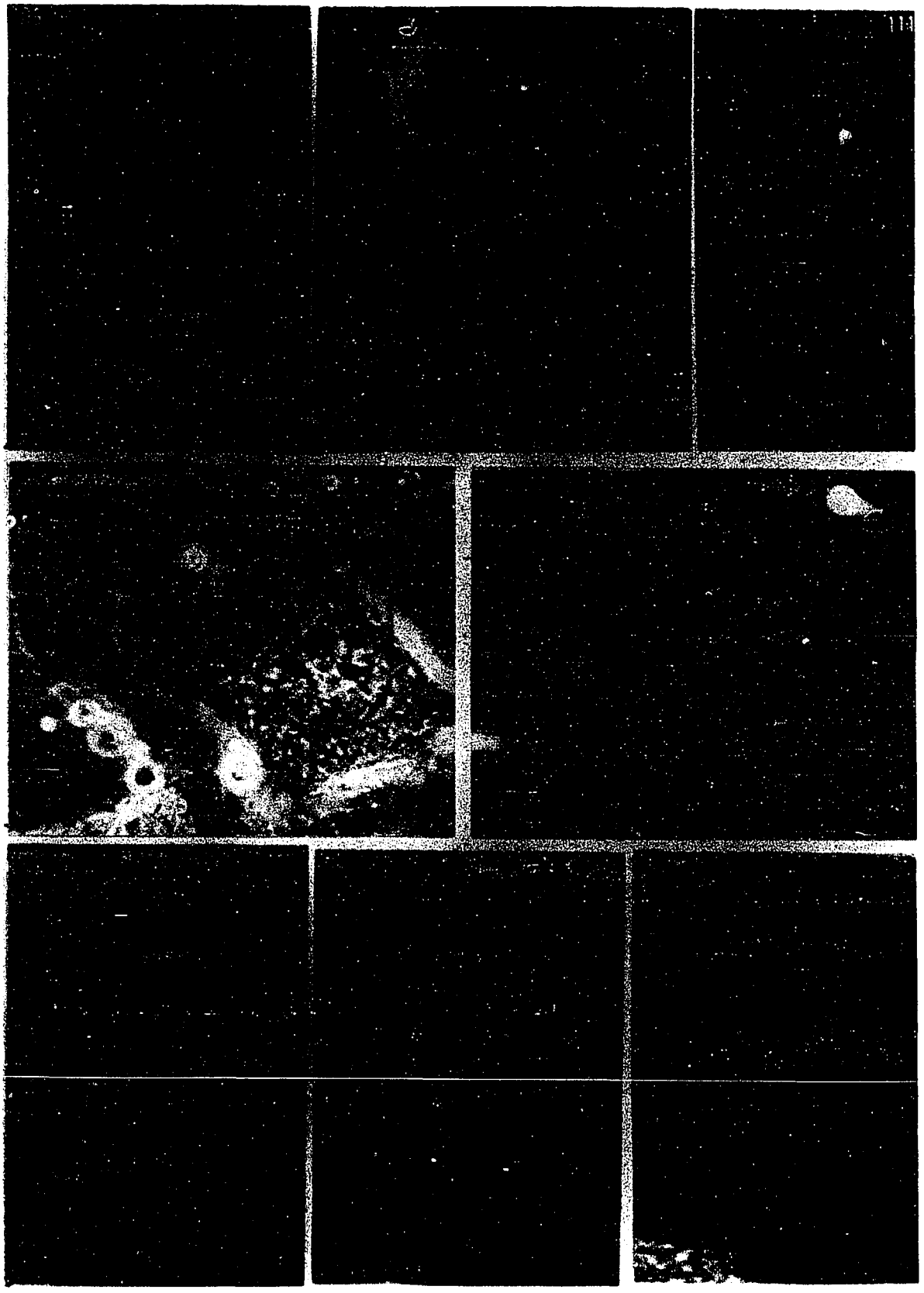

Plate XVI

Figure 117. Portions of a group of L-fibroblasts fixed in 4% buffered glutaraldehyde in which mitochondria (M) and Golgi granules (G) are preserved. A few cytoplasmic vacuoles (Va) are in evidence. Nuclei are limited by a discrete nuclear membrane. The karyoplasm contains several nucleoli but poorly resolvable chromatin. DMP 1317X

Figure 118. The same cells as in Figure 117 showing the relatively highest BP accumulations in Golgi granules (G). No demonstrable BP uptake is displayed by cytoplasmic vacuoles (V), mitochondria (M) or by any nuclear structure (N). UVFM 1317X

Figure 119. A stellate L-fibroblast fixed in Lewitsky's fluid showing relatively high levels of BP accumulation in frank lipid droplets (L). No apparent BP uptake is displayed by cytoplasmic vacuoles (Va) or by any nuclear structure (N) except possibly the nuclear membrane (Nm). UVFM 1317X

Figure 120. The same cell as in Figure 119 showing a highly refractile nucleus surrounded by a discrete, intact nuclear membrane (Nm). In the cytoplasm, vacuoles (Va) and frank lipid droplets (Ld) occur within the coarsely precipitated protoplasm. DMP 1317X

Figure 121. Several fibroblasts viewed with the ordinary light microscope which have been air-dried on crystalline beds of BP. The nuclear region appears as a clear central space (N). Coagulation of material is prominent at the cells' surfaces. Disjunction of the cytoplasmic processes (Cp) is occasional. OLM 1317X

Figure 122. The same dehydrated cells as in Figure 121 fixed

in buffered OsO_4 after exposure to BP. The central nuclear regions (N) show no fluorescence. Fluorescence intensity is greatest in the material deposited in association with the cell surface. UVFM 1317X

Figure 123. Portions of several cells fixed in buffered OsO_4 and viewed with ordinary light microscopy. Reduced osmium is notable in the Golgi zones (Gz) and in the Golgi granules (G) in the cell cytoplasm. OLM 1317X

Figure 124. Two Zenker's fluid-fixed fibroblasts, posted-treated for the removal of mercury, allowed to air-dry on clean glass slides. Autofluorescence is seen to be absent from the vicinity of the nucleus (N) and most intense in the vicinity of the cell surface. UVFM 1317X

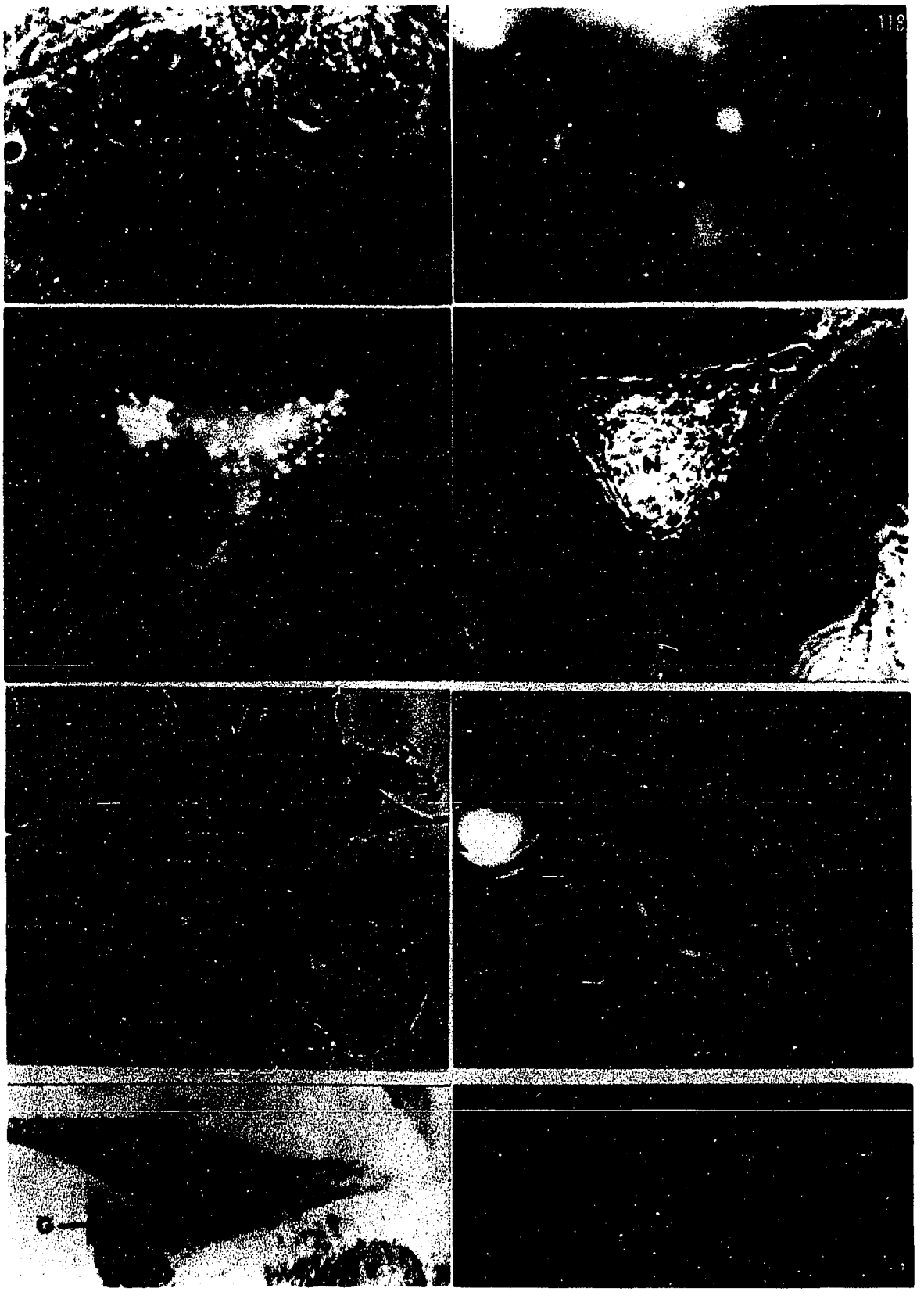

Plate XVII

Figure 125. An L-929 fibroblast frozen slowly in a refrigerator (approximately -15°C), fixed in Lillie's HCHO and treated with DNAase. The nuclei were Feulgen positive. The cells show severe damage, the nuclear membrane (Nm) being broken, isolated nuclei free of cytoplasm float in the medium (N) and free or sessile vesicles (V) appear. DMP 1317X

Figure 126. The same damaged cell as in Figure 125 showing a lack of BP uptake in the damaged nucleus or in the isolated nucleus (N) and in the free vesicle (V). Nuclear rims of the intracellular nucleus (Nm) and the isolated nucleus and the walls of the free vesicle show demonstrable BP uptake. UVFM 1317X

Figure 127. Several cells fixed and extracted in 5% TCA for 1 1/2 hours at room temperature. The nuclei were Feulgen positive. BP accumulation in the fine cytoplasmic processes (Fc) is approximately comparable with the levels in the general cytoplasmic matrix. No demonstrable BP is apparent in cytoplasmic vacuoles (Va) or in any nuclear structure (N). UVFM 1317X

Figure 128. The same cells as in Figure 127 showing TCA preservation of the fine cytoplasmic processes (Fp). The cytoplasm of the cells is filled with coarsely precipitated material and a few vacuoles (Va). A coarse precipitate of karyoplasm fills the nuclei (N) which also contain discrete nucleoli and nuclear membranes which appear intact only in the upper cell. DMP 1317X

Figure 129. An EtOH-fixed fibroblast extracted for 2 1/2

hours at 60°C with 1M NaCl. No Feulgen staining was obtained from the nuclei of these preparations. A number of vacuoles (V) occur in the coarsely precipitated cytoplasm. The nuclear membrane is broken at many points. Discrete nucleoli (nc) are situated in a coarsely precipitated karyoplasm. DMP 1317X

Figure 130. The same cell as in Figure 129 showing distinct BP accumulation in apparent nucleolar structures (nc). The cytoplasmic vacuoles (v) and the rest of the nuclear region show no BP accumulation. UVFM 1317X

Figure 131. Portions of a group of fibroblasts fixed and extracted in 5% TCA for 5 hours at 60°C. All the preparations were Feulgen positive. DMP 1317X

Figure 132. The same group of cells as in Figure 131 showing no apparent BP uptake by any nuclear structure in these TCA-extracted cells. UVFM 1317X

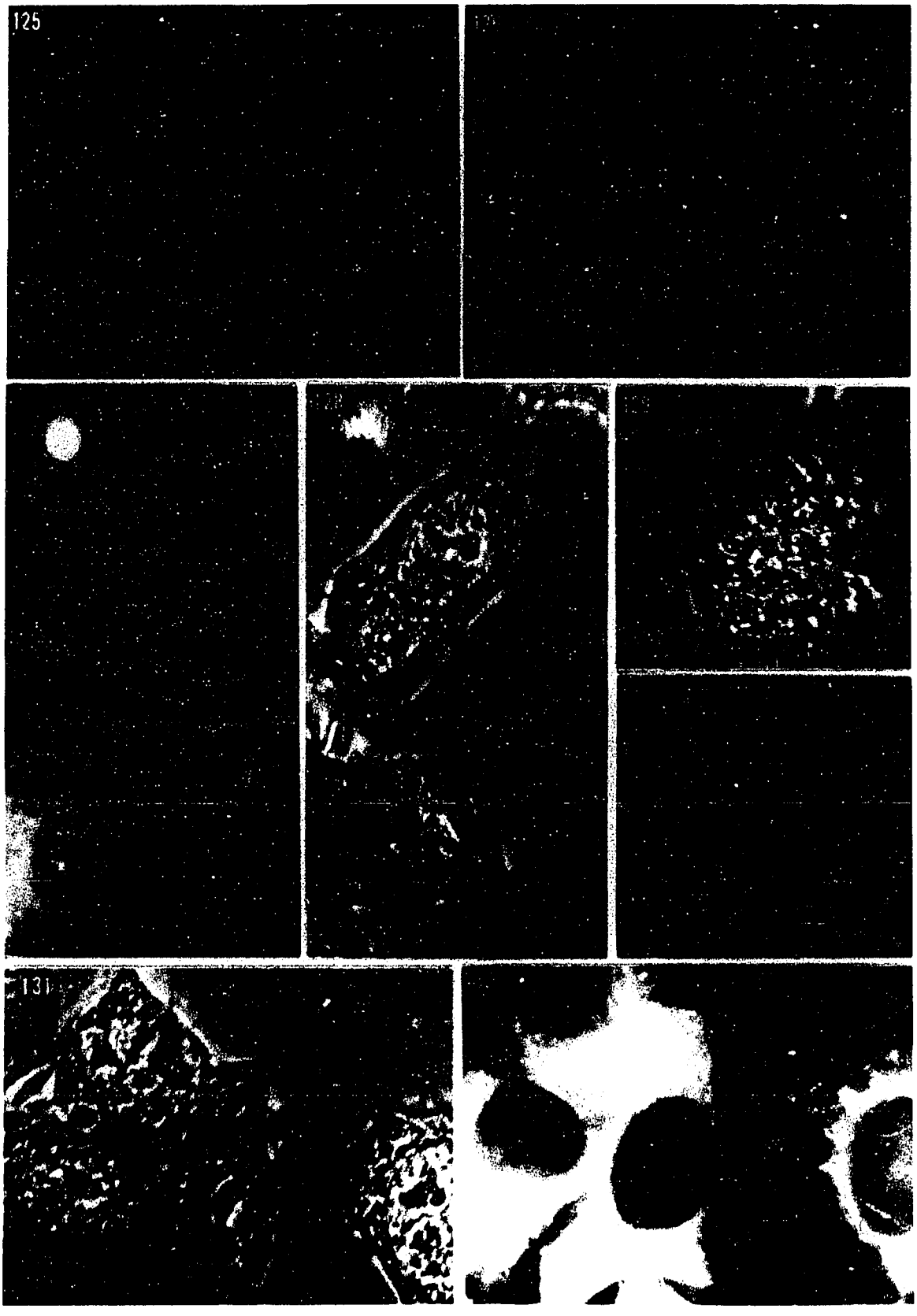
125
131

Plate XVIII

Figure 133. An L-929 fibroblast fixed in Zenker's fluid and post-treated for removal of mercury followed by incubation in RNAase. The general region of a Golgi zone (Gz) is among the features of the cytoplasm. The nucleus contains fine chromatin material, nucleoli (nc) and a distinct nuclear membrane. DMP 1317X

Figure 134. The same cell as in Figure 133 showing relatively higher BP accumulation in the Golgi zone (Gz) than in the general cytoplasmic matrix. All nuclear structures, including chromatin, nucleoli and the nuclear membrane show no demonstrable BP accumulation. UVFM 1317X

Figure 135. An L-fibroblast digested in RNAase and unstained by Gallocyanin-Chrom Alum anywhere in the cytoplasm. Relatively higher BP concentrations are localized in the Golgi granules (G) and no discernible BP uptake occurs in cytoplasmic vacuoles (Va) or any nuclear structure (N). The small fluorescence regions overlying the nucleus are thought due to cytoplasmic structures in the sub-or supranuclear cytoplasm. UVFM 1317X

Figure 136. The same cell as in Figure 135 showing cytoplasmic vacuoles (Va) and Golgi granules (G) and nuclei containing chromatin (Hc), several nucleoli and a well-defined nuclear membrane. DMP 1317X

Figure 137. A mid-anaphase fibroblast showing a suggestion of BP accumulation in the interzonal spindle region (I) but no demonstrable CH uptake in the daughter chromosomes (C). UVFM 1317X

Figure 138. The same cell as in Figure 137 fixed in Zenker's fluid, post-treated for removal of mercury and incubated in RNAase so that all cytoplasmic staining by Gallocyanin-Chrom Alum was abolished. Spindle fibers are visible in the interzonal region (I). DMP 1317X

Figure 139. An L-929 fibroblast in late anaphase after hydrolysis with DNAase. The daughter chromosomes (C) are outlined by the dark perichromosomal cytoplasm. No distinct interzonal spindle fibers are discernible in the general interzonal region (I). DMP 1317X

Figure 140. The same late anaphase cell digested with RNAase as in Figure 139. Levels of BP accumulation in the general interzonal spindle region (I) are comparable with those in the non-spindle cytoplasm. No demonstrable BP uptake by any of the daughter chromosomes is apparent. (C). UVFM 1317X

Figure 141. An L-929 fibroblast beginning telophase; the mitotic cell has been fixed in Zenker's fluid, post-treated for removal of mercury and incubated with RNAase until all cytoplasmic staining with Gallocyanin-Chrom Alum has been abolished. Daughter chromosomes are seen in the process of converting to karyomeres (C). DMP 1317X

Figure 142. The same early telophase cell as in Figure 141. After the RNAase depletion of cellular RNA, the karyomeres show no evidence of BP accumulation (C). UVFM 1317X

Figure 143. A group of L-fibroblasts rapidly frozen in isopentane vapors, fixed in Lillie's HCHO and digested with DNAase. No cytoplasmic staining with Gallocyanin-Chrom Alum was observed in these

preparations. Nuclear chromatin is easily discernible along with several nucleoli and an intact nuclear membrane (Nm). A few cytoplasmic vacuoles (Va) are seen in the central cytoplasmic region. In the hyaline peripheral cytoplasm, discrete fibrous material is clearly preserved (F). DMP 1317X

Figure 144. The same RNAase-treated cell as in Figure 143 showing a relative BP uptake by the fibrous material (F) that is comparable to that in the general cytoplasmic matrix. Cytoplasmic vacuoles (Va) and all nuclear structures (N) show no demonstrable BP accumulation. Faint CH-fluorescence over the nuclear region is interpreted to be due to over- or underlying cytoplasmic structures. UVFM 1317X

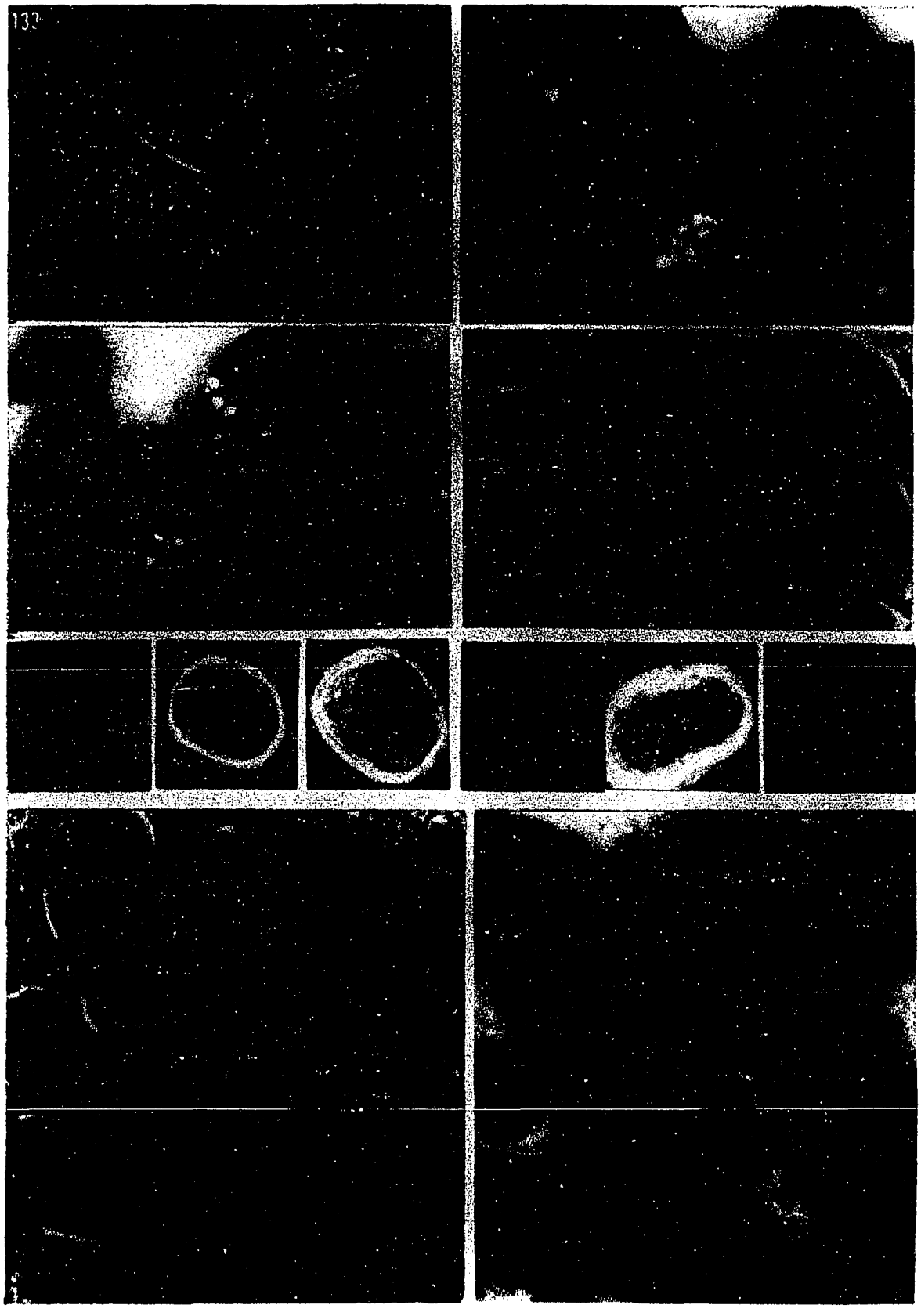
133

Plate XIX

Figure 145. A pair of Zenker's-fixed fibroblasts treated for removal of mercury and digested in DNAase without the presence of the magnesium co-factor in the incubation medium. None of the preparations contained intranuclear Feulgen-stainable material. These cells apparently lacking, or low in, DNA, show no BP accumulations in any nuclear structures. Golgi zones (Gz) and Golgi granules (G) show relatively high BP concentrations. UVFM 1317X

Figure 146. The same DNA-extracted cells as in Figure 145. Cytoplasmic Golgi zones (Gz) and Golgi granules (G) are seen. Nuclei apparently still retain chromatin residues, nucleoli and distinct nuclear membranes. DMP 1317X

Figure 147. Portions of several L-fibroblasts incubated in complete DNAase incubation medium; preparations are Feulgen-negative. Fine cytoplasmic processes are preserved (Fp), as are Golgi granules (G), nucleoli (nc) and distinct nuclear membranes (Nm) surrounding the chromatin-laden non-nucleolar karyoplasm. DMP 1317X

Figure 148. The same cells as in Figure 147 from which DNA has apparently been extracted. Relatively high BP uptake is seen in the Golgi granules (G). Uptake of BP by the fine cytoplasmic processes (Fp) is comparable with that in the general cytoplasmic matrix. No demonstrable accumulation of BP by any nuclear structure is in evidence. UVFM 1317X

Figure 149. A cell from a control preparation involving treatment in an incubation medium lacking DNAase for 6 hours at 60°C. The

preparation stains with the Feulgen reaction. Golgi granules (G) show the relatively highest intracellular accumulations of the CH, while cytoplasmic vacuoles (Va) and all nuclear structures show no demonstable BP uptake (N). UVFM 1317X

Figure 150. The same cell as in Figure 149 showing Golgi granules (G), cytoplasmic vacuoles (Va) and nuclear structures (N) at a low focal plane. DMP 1317X

Figure 151. A mid-anaphase fibroblast incubated in DNAase and part of a preparation which was unstained by the Feulgen reaction. No demonstrable CH accumulation is seen in the daughter chromosomes from which all apparent DNA has been removed (C). The interzonal spindle region (I) shows a slightly lower relative accumulation of BP than the non-spindle cytoplasm. UVFM 1317X

Figure 152. The same anaphase cell as in Figure 151 showing daughter chromosomes (C) and some spindle fibers in the interzonal region (I). DMP 1317X

Figure 153. A large L-929 fibroblast in later anaphase in preparations treated with DNAase and unstained by the Feulgen reaction. The chromosomal mass is phase light (C) and surrounded by phase-dark perichromosomal cytoplasm. Fibers are seen (IF) in the interzonal spindle region stretched between the poleward oriented daughter chromosomes. DMP 1317X

Figure 154. The same cell as in Figure 153 showing no apparent uptake of BP in anaphase chromosomes from which DNAase has been removed (C). The BP concentration in the general interzonal spindle region is

similar to that of the non-spindle cytoplasm. Spindle fibers are not visible re their uptake of BP. UVFM 1317X

Figure 155. An L-929 fibroblast in metaphase in preparations of DNAase-treated cells which were unstained with the Feulgen reaction. No demonstrable BP uptake is visible in the chromosomal material on the metaphase plate (C). Fine cytoplasmic processes (Fp) show distinct BP uptake. Granules showing relatively high BP accumulations in the peripheral cytoplasm are Golgi granules. UVFM 1317X

Figure 156. The same cell as in Figure 155 showing metaphase chromosomes (C) and fine cytoplasmic processes (Fp). DMP 1317X

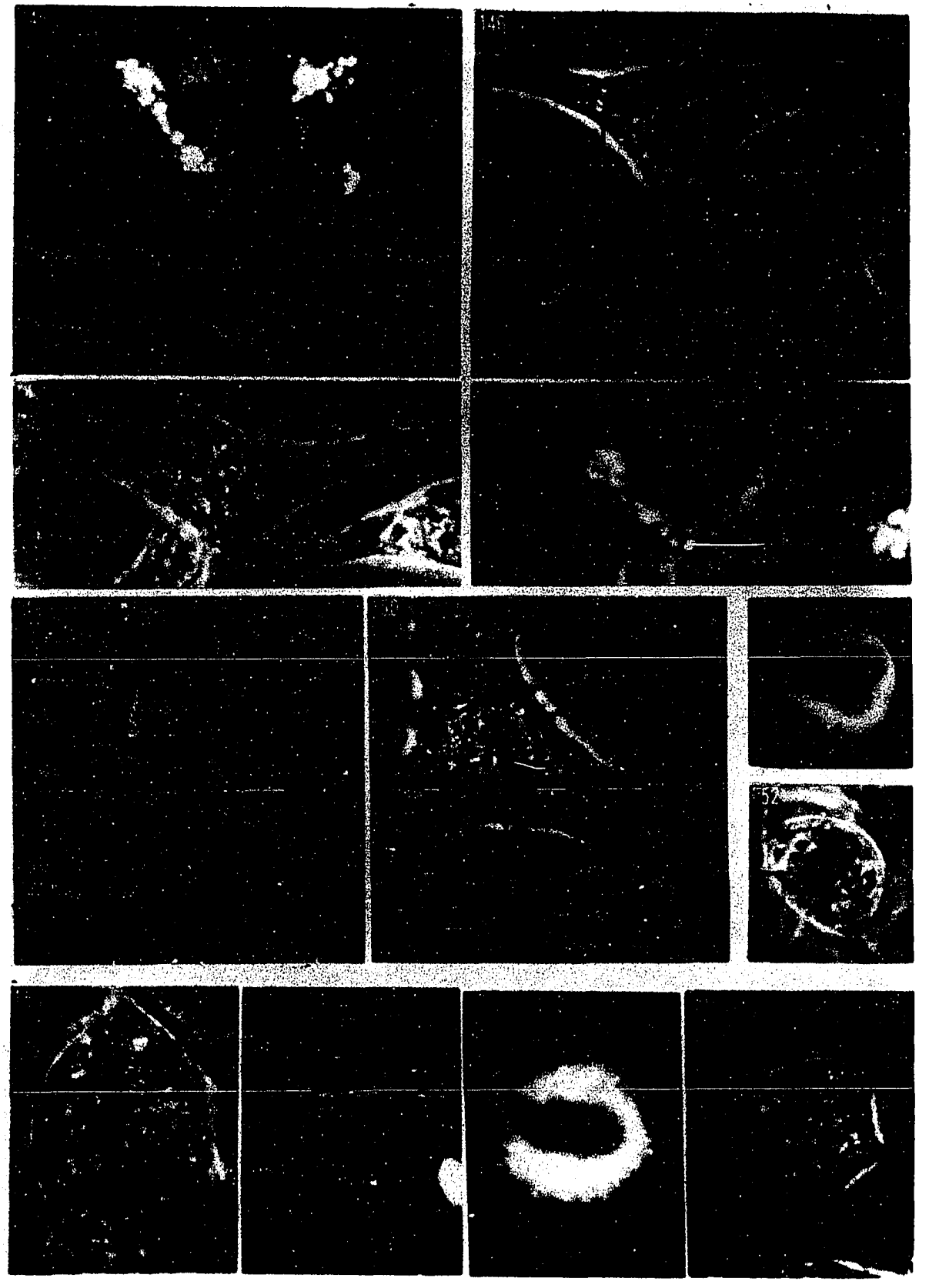

Plate XX

Figure 157. An L-fibroblast from a preparation digested with DNAase in complete incubation medium, except for the absence of chloramphenicol; Feulgen reactions on these preparations were negative. Golgi granules (G) and cytoplasmic vacuoles are seen as well as distinct nucleoli, chromatin material and a nuclear membrane (Nm). Coccus-type bacteria are seen in the media around the cell (Bac). DMP 1317X

Figure 158. The same cell as in Figure 157 showing no demonstrable BP in any nuclear structure (N). Golgi granules (G) show the relatively highest BP accumulations in the cytoplasm. BP uptake by the contaminating bacteria is not in evidence. UVFM 1317X

Figure 159. An elongated L-929 fibroblast incubated in McIlvaine's buffer (pH 6.0) for 6 hours at 37^{o}C; the preparations were Feulgen-positive. Fixation was in Zenker's fluid, sequentially followed by mercury removal with immersion in Lugol's solution and thiosulfate solutions. Golgi granules (G) are seen in the cytoplasm and a distinct nuclear membrane (Nm) delimits the nucleus (N). DMP 1317X

Figure 160. The same cell as in Figure 159 showing no demonstrable BP uptake by intranuclear structures (N). Relatively higher BP accumulations occur in the Golgi granules (G) and in the region of the nuclear perimeter (Nm), which is thickened due to a coagulation of cytoplasmic and nuclear material in the region of the nuclear membrane

and thus not interpretable as a true accumulation of BP in the nuclear membrane. UVFM 1317X

Figure 161. A Zenker's-fixed L-fibroblast, post-treated for removal of mercury and successively digested in incubation media containing DNAase and RNAase; Feulgen-staining is absent in these preparations and staining with Gallocyanin-Chrom Alum is reduced to a faint, unlocalized bluish color. Golgi granules (G) and cytoplasmic vacuoles (Va) are seen. In the nucleus, chromatin (Hc) and some nucleoli (as phase-light structures) are visible. DMP 1317X

Figure 162. The same cell as in Figure 161, digested with DNAase and RNAase sequentially. The relatively highest accumulations of BP in the cytoplasm are localized in the Golgi granules (G), while no demonstrable BP uptake is seen in the cytoplasmic vacuoles (Va) or in a nuclear structure (N). UVFM 1317X

Figure 163. A pair of L-cells, Zenker-fixed post-treated for removal of mercury and sequentially incubated in media containing DNAase and RNAase, so that no Feulgen staining or only light Gallocyanin-Chrom Alum staining is seen. A Golgi zone (Gz) is seen preserved in the left hand cell. Nuclei of these cells from which both DNA and RNA have been extracted contain well-defined nucleoli (nc), fine chromatin material and a distinct nuclear membrane (Nm). DMP 1317X

Figure 164. The same DNAase and RNAase-treated cells as in Figure 163 showing no demonstrable BP accumulation in any nuclear structure (N) but very high relative BP concentrations in the Golgi zone (Gz). UVFM 1317X

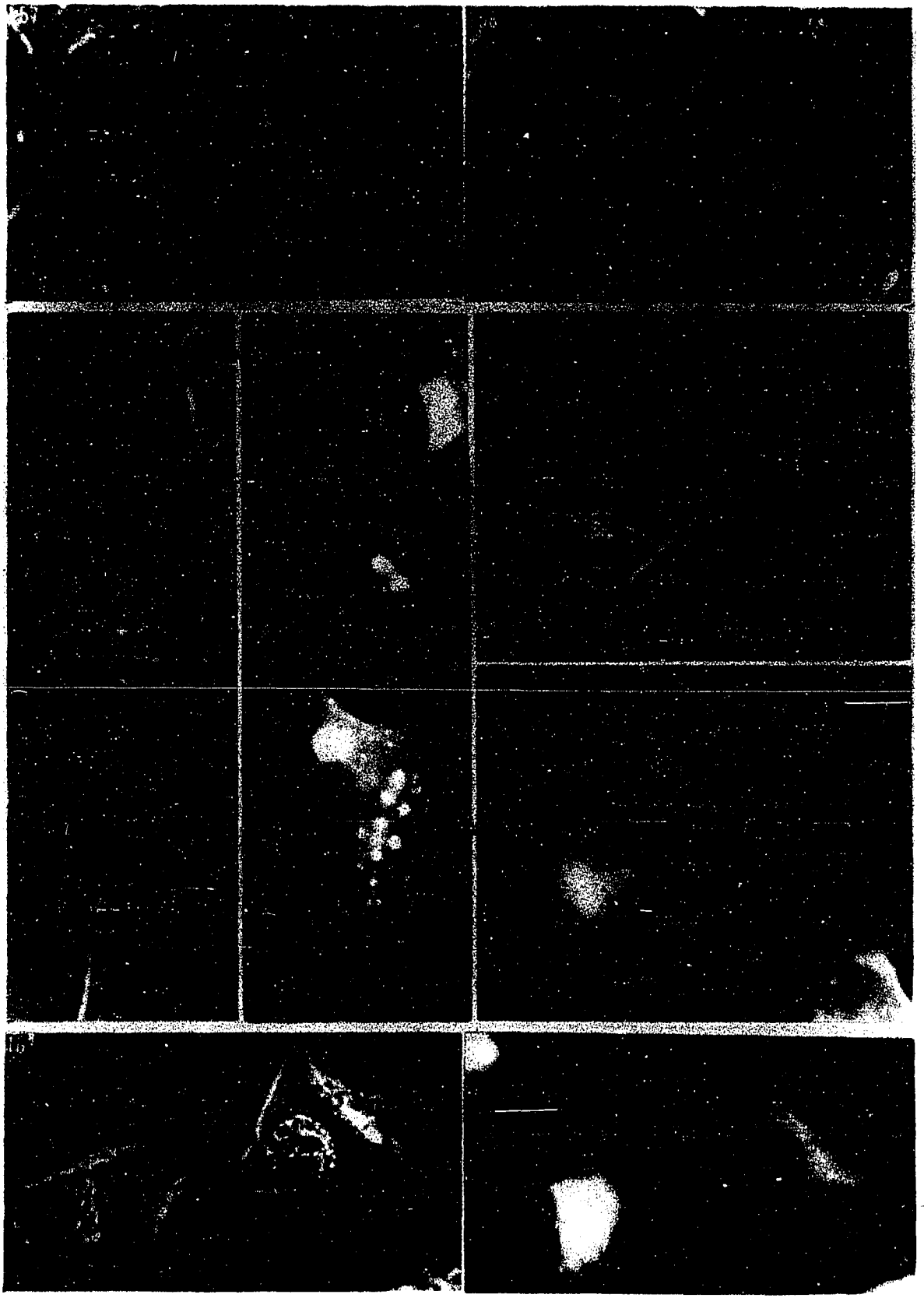

Plate XXI

Figure 165. Portions of several L-929 fibroblasts fixed in Bouin's fluid after being grown on BP beds in vitro. A Golgi zone (Gz) is seen preserved in the cytoplasm of the left hand cell. The nuclear membrane (Nm) is thickened by coagulation of surrounding material. Distinct nucleoli (nc) appear in the coarsely precipitated non-nucleolar karyoplasm. DMP 1317X

Figure 166. The same Bouin's-fixed L-cells as in Figure 165 showing no demonstrable BP accumulation in any nuclear structure (N). The relatively highest accumulation of BP in the cytoplasm is localized in the Golgi zone (Gz). UVFM 1317X

Figure 167. Portions of several L-cells fixed in Bouin's fluid and extracted with the lipid solvent $CHCl_3$:MeOH for five hours; the cells had not been grown on BP beds in vitro. U-V-fluorescence photomicrographs made of this same cell (not shown) did not display any visible fluorescence. The nuclear membrane (Nm) is seen to have lost much of its integrity. Nucleoli (nc) are preserved. DMP 1317X

Figure 168. Portions of several Bouin's-fixed cells extracted with $CHCl_3$:MeOH for five hours; the cells had been grown on BP beds in vitro. The nuclear membrane (Nm) is continuous around the nuclear perimeter. No fluorescence was visible in photomicrographs taken with u-v light (not shown). DMP 1317X

Figure 169. A Bouin's-fixed L-cell extracted with $CHCl_3$:MeOH for 12 hours; the cells were not grown on BP in vitro prior to

fixation. The nuclear membrane (Nm) is seen to be discontinuous over much of its course. No fluorescence was visible in this cell under u-v-fluorescence microscopy. DMP 1317X

Figure 170. A Bouin's fixed L-cell extracted with $CHCl_3$:MeOH for 12 hours; the cell had been grown on BP in vitro prior to fixation. The nuclear membrane is seen to be continuous throughout its course around the nuclear perimeter. No fluorescence was visible within the cell using u-v-fluorescence microscopy. DMP 1317X

Figure 171. A Bouin's-fixed L-cell extracted with $CHCl_3$:MeOH for approximately 20 hours; the cell had not been grown on BP in vitro prior to fixation and extraction. The nuclear membrane is absent from the margin of much of the nucleus, while nucleoli (nc) are preserved. Cytoplasmic vacuoles (Va) are seen. A break in a cell process (Cp) occurs on the right side of the cell. DMP 1317X

Figure 172. The same cell as in Figure 171 showing the autofluorescence of the cell throughout the cytoplasm, except for the cytoplasmic vacuoles (Va), and in residual nucleolar structures (nc). UVFM 1317X

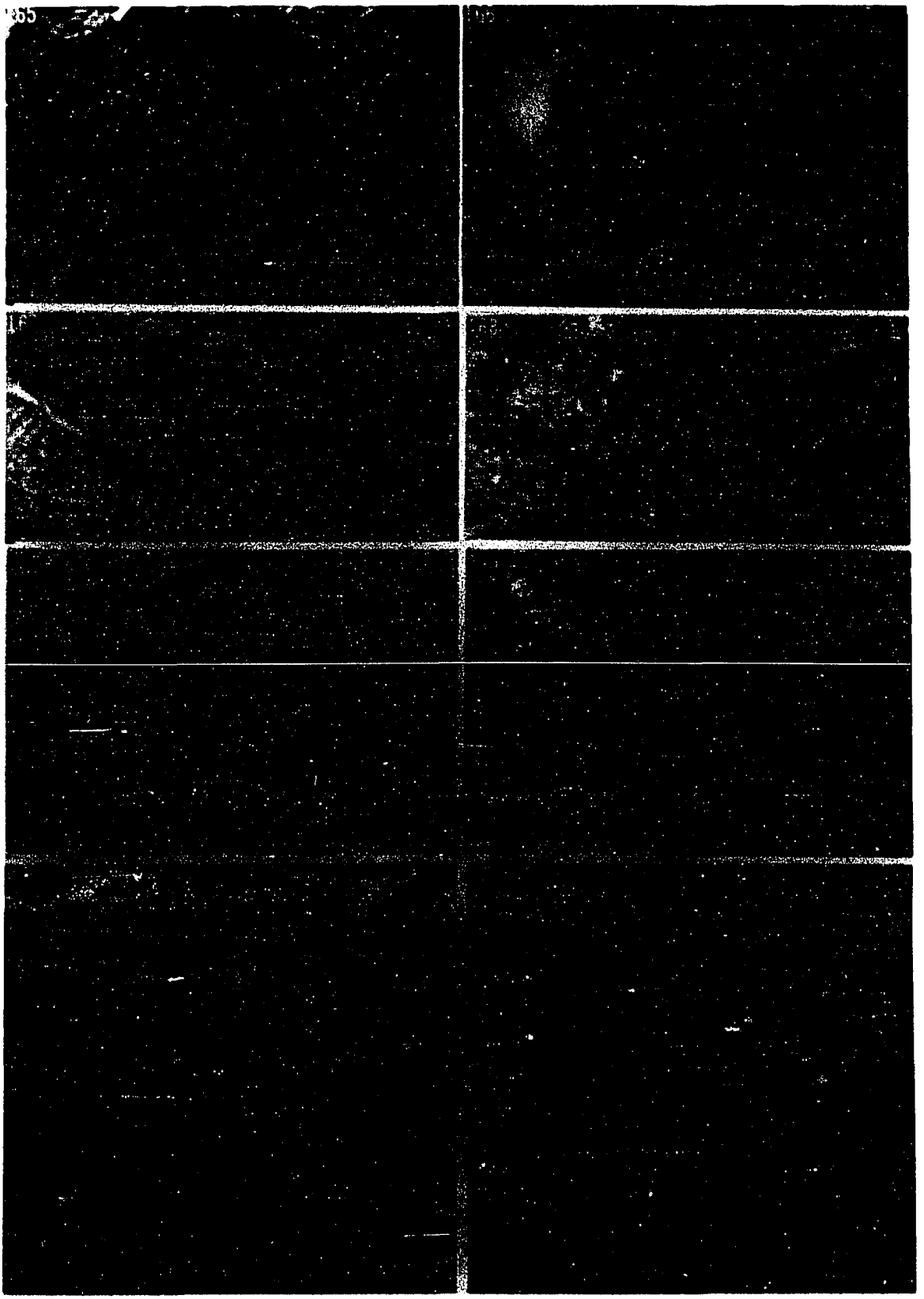

Plate XXII

Figure 173. Several L-fibroblasts fixed and extracted in $CHCl_3$:MeOH 3-5 hours after prior growth in vitro on a crystalline bed of BP. The nuclear membrane, nucleoli (nc) and fine chromatin material are preserved. Cytoplasmic processes (Cp) are intact. DMP 1317X

Figure 174. The same cells as in Figure 173 showing unextracted BP. BP-fluorescence is visible in nucleolar structures (nc) as well as throughout the general cytoplasmic matrix. UVFM 1317X

Figure 175. An L-929 fibroblast fixed and extracted with $CHCl_3$:MeOH for five hours; the cells had not been grown on, or otherwise exposed to, BP in vitro prior to, or during, fixation and extraction. The integrity of the nuclear membrane (Nm) is seen to have been lost. DMP 1317X

Figure 176. An L-929 fibroblast fixed and extracted with $CHCl_3$:MeOH for five hours; the cells had been grown on BP in vitro prior to fixation. No fluorescence was observed with u-v-fluorescence microscopy or photomicrography in this cell. The nuclear membrane (Nm) is seen to be intact around the nuclear perimeter. DMP 1317X

Figure 177. An L-929 fibroblast fixed and extracted with $CHCl_3$:MeOH for 20 hours; the cell had not been grown on BP in vitro prior to fixation. The nuclear membrane is not discernible. A number of cytoplasmic vesicles (Va) are seen in the central cytoplasmic region. DMP 1317X

Figure 178. Low power u-v-fluorescence photomicrographs of a preparation similar to the one viewed in Figure 177. The autofluorescence of these cells is too light to be photographed with high-power

magnifications. The cells were not grown on BP in vitro prior to fixation and extraction. UVFM 337X

Figure 179. An L-929 fibroblast fixed and extracted with $CHCl_3$:MeOH for seven days in a Soxhlet apparatus after being grown on a crystalline bed of BP in vitro. The cytoplasm and general karyoplasm are composed of a coarse precipitate. Nucleoli (nc) are seen in the nucleus, and the nuclear membrane is broken at a few points in its course encircling the nucleus. DMP 1317X

Figure 180. The same cell as in Figure 179 showing the localization of autofluorescence in the cytoplasm and the nucleoli of the $CHCl_3$:MeOH-extracted fibroblast. UVFM 1317X

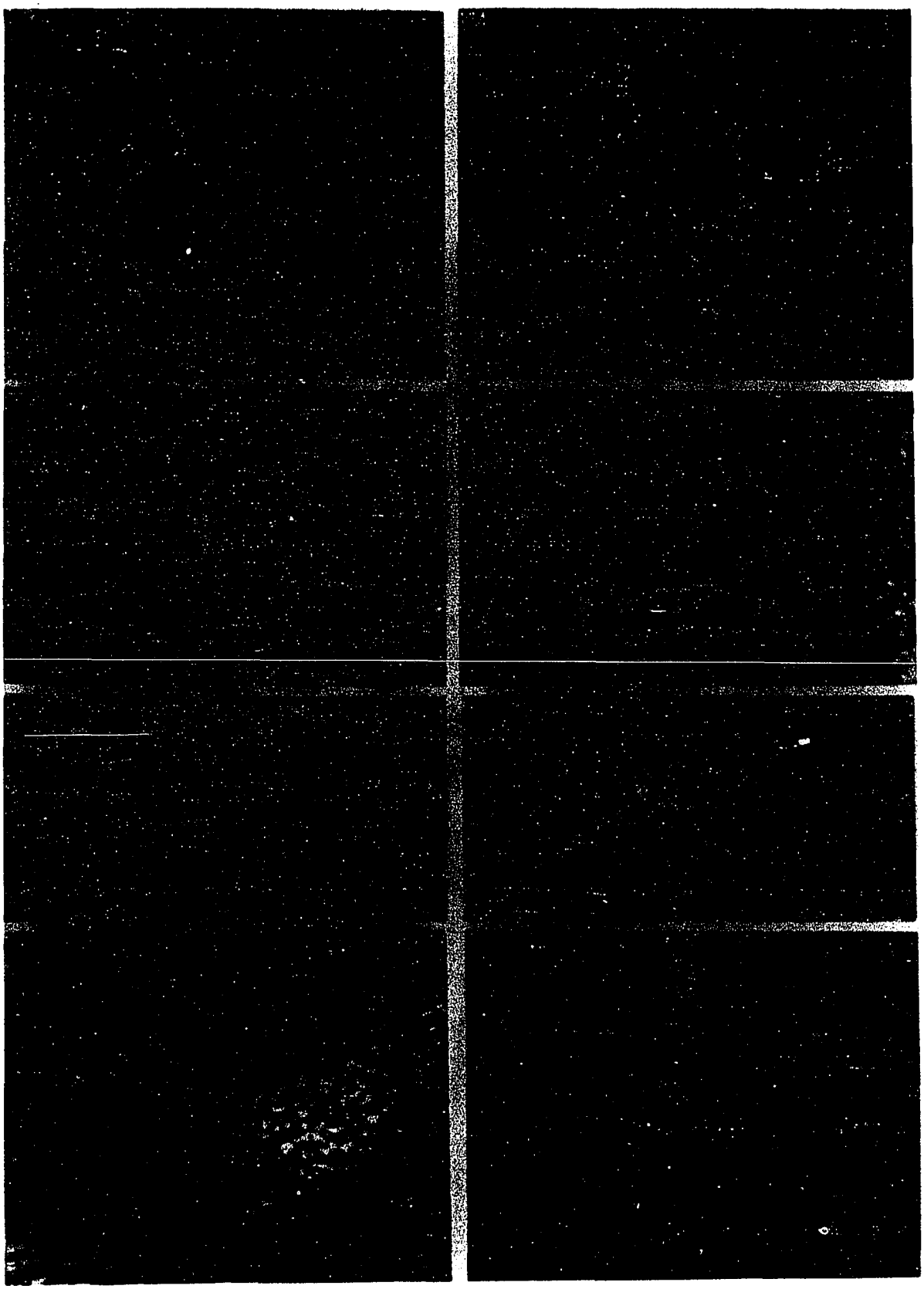

Plate XXIII

Figure 181. Two $CaCl_2$-HCHO-fixed fibroblasts extracted with $CHCl_3$:MeOH for five hours; the cells had been grown on BP crystals prior to fixation. A Golgi zone (Gz) has been preserved. DMP 1317X

Figure 182. Cells from the same preparation as those in Figure 181. The level of CH-fluorescence was too low for high-magnification photomicrography. This low power photomicrograph is used to show the faint, but discernible residual BP in these lipid-extracted cells. UVFM 337X

Figure 183. A $CaCl_2$:HCHO-fixed L-cell extracted with $CHCl_3$:MeOH for 12 hours; the cell had <u>not</u> been exposed to BP. No visible fluorescence was found in the cell. Nucleoli (nc) are preserved, but the nuclear margin shows signs of damage. The non-nucleolar Karyoplasm (Ky) shows faintly discernible chromatin. DMP 1317X

Figure 184. A $CaCl_2$-HCHO-fixed L-cell extracted with $CHCl_3$:MeOH for 12 hours; the cell had been grown on crystalline BP <u>in vitro</u>. No visible fluorescence was encountered in the cell using u-v-fluorescence microscopy. The right hand cell shows extensive cytoplasmic vacuolation (Va). DMP 1317X

Figure 185. A $CaCl_2$-HCHO-fixed L-fibroblast extracted with $CHCl_3$:MeOH for 24 hours; the cell had <u>not</u> been grown on BP <u>in vitro</u>. Structural damage along the nuclear margin has occurred, but it is not extensive. DMP 1317X

Figure 196. A low power u-v-fluorescence photomicrograph of the same preparation as in Figure 185. The light autofluorescence

quenched in approximately one minute of continous u-v irradiation. Differential localization of the autofluorescence is not apparent in the cells (X) at this magnification. UVFM 1317X

Figure 187. A group of $CaCl_2$:MeOH-fixed L-cells extracted with $CHCl_3$:MeOH for 24 hours; the cells had been grown in vitro on crystalline BP. Nuclear structures preserved are: nucleoli, the nuclear rim and the non-nucleolar karyoplasm in which chromatin is indistinctly resolvable. DMP 1317X

Figure 188. A low-power u-v-fluorescence photomicrograph of the same preparation as in Figure 187. The dim autofluorescence quenched within one minute of continuous u-v irradiation and could only be photographed at low magnifications. It is notable that the fluorescence intensity seems greatest in the nuclear region of the cells (X). UVFM 1317X

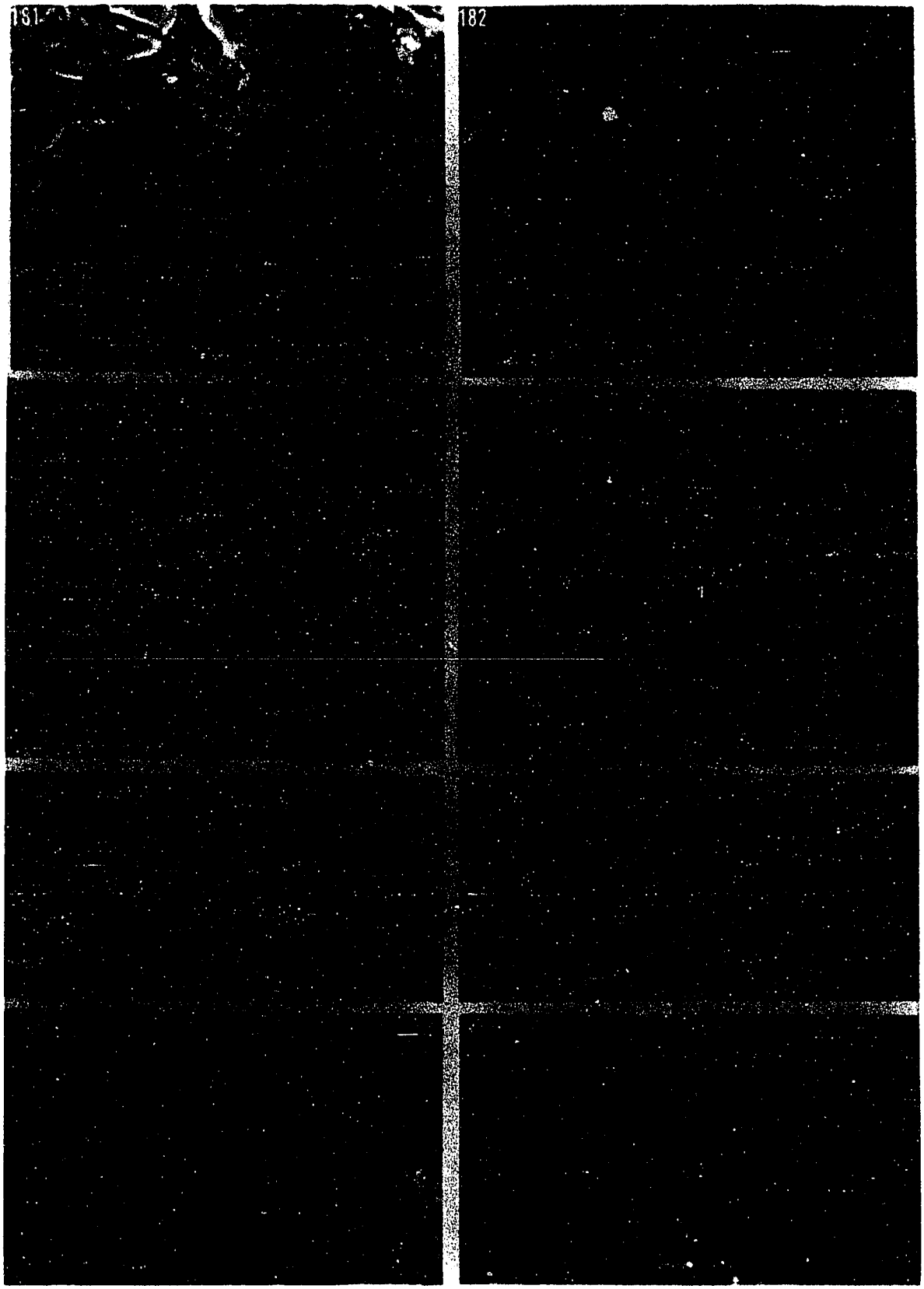
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Plate XXIV

Figure 189. A low power u-v-fluorescence photomicrograph of L-cells (X) grown in vitro on BP crystals, fixed in 95% EtOH one hour and extracted with $CHCl_3$:MeOH for five hours. Levels of residual CH-fluorescence were too low for photomicrography at higher magnifications. UVFM 337X

Figure 190. A picture of L-fibroblasts (X) demonstrating, as in Figure 189, the presence of fluorescent material, presumbly BP, after EtOH-fixation and $CHCl_3$:MeOH extraction for 5 hours on fibroblasts grown on BP in vitro. The fluorescence levels were too faint for photography at higher magnifications. UVFM 337X

Figure 191. An EtOH-fixed fibroblast extracted with $CHCl_3$: MeOH for 12 hours; the cell had not been grown on BP. No fluorescence was displayed on u-v-fluorescence photomicrographs exposed for long periods of time. A Golgi zone (Gz) is seen adjacent to the nuclear membrane (Nm). A fine precipitate fills the nucleus (Ky). Fine cytoplasmic processes are still intact (Fp). DMP 1317X

Figure 192. An EtOH-fixed fibroblast extracted with $CHCl_3$:MeOH for 12 hours; the cell had been grown on BP. No fluorescence was displayed by the cell either by direct observation or on u-v-fluorescence photomicrographs. The nuclear membrane (Nm) is a continuous structure surrounding the nucleus (compare with the nuclear membrane of the cell in Figure 191). DMP 1317X

Figure 193. An EtOH-fixed fibroblast extracted with $CHCl_3$:MeOH for 24 hours; the cell had not been grown on BP in vitro. A nucleolus

(nc) is discernible in the nucleus whose limiting membrane (Nm) shows extensive discontinuities. DMP 1317X

Figure 194. The same cell as in Figure 193 showing the autofluorescence of the cell extracted for 24 hours in $CHCl_3$:MeOH. The autofluorescence is general in the cytoplasm and in the nucleolus (nc). UVFM 1317X

Figure 195. A spindle-shaped L-fibroblast fixed in EtOH and extracted with $CHCl_3$:MeOH for 7 days in a Soxhlet apparatus; the cell had been grown on BP in vitro. A residual nucleolus can be seen centrally situated in the nucleus (nc), and the nuclear membrane shows an extensive loss of continuity (Nm). DMP 1317X

Figure 196. The same cell as in Figure 195 showing autofluorescence throughout the cytoplasm and in the nucleolar region (nc). UVFM 1317X

Figure 197. A spindle-shaped L-fibroblast fixed in EtOH and extracted with $CHCl_3$:MeOH displaying a residual nucleolus (nc) in a refractile nucleus whose limiting membrane is broken in several places (Nm). The cell had not been grown on BP in vitro. DMP 1317X

Figure 198. The same cell in Figure 197 showing autofluorescence in the cytoplasm and in the nucleolar region (nc). UVFM 1317X

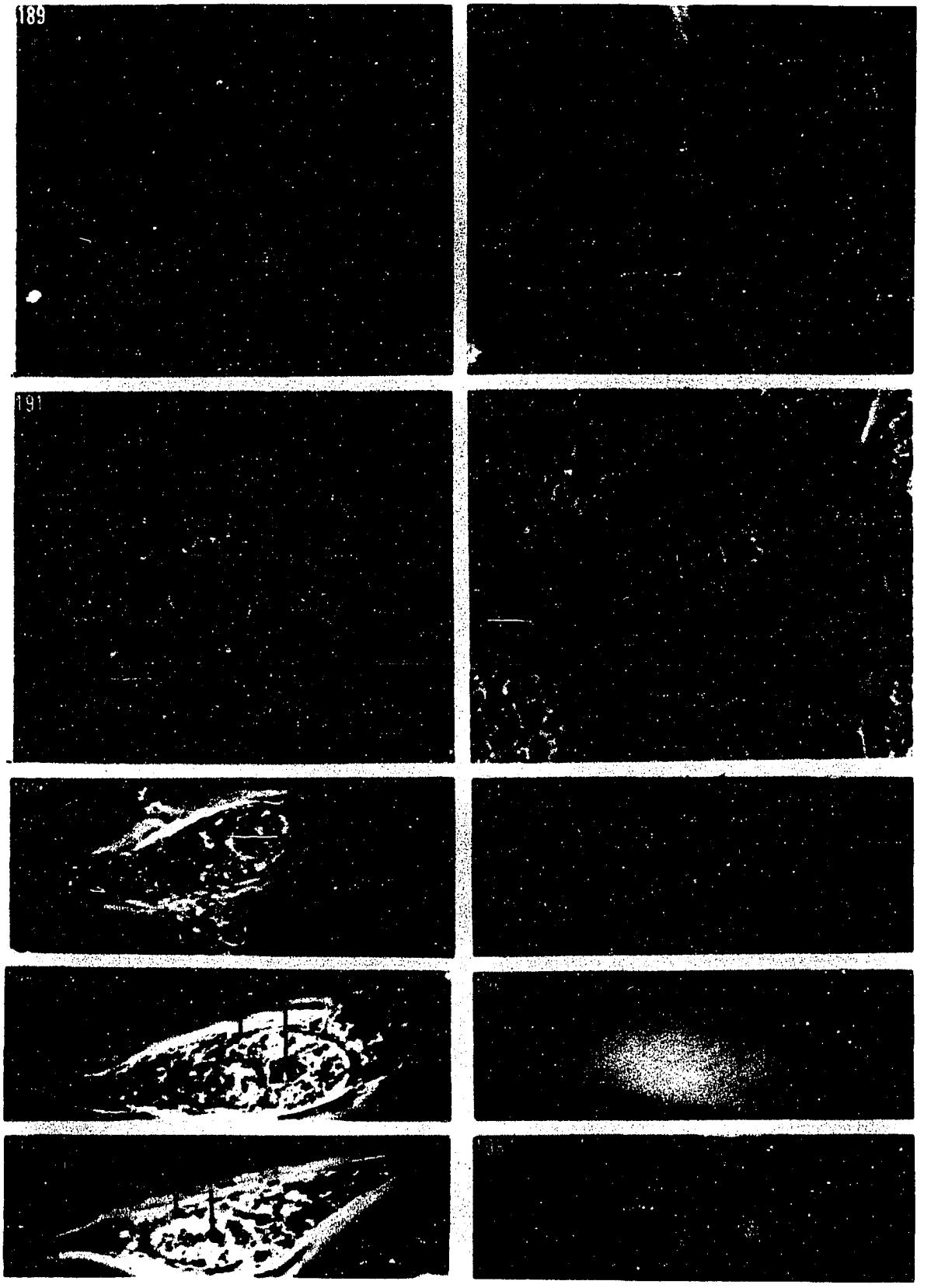
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Plate XXV

Figure 199. A group of several L-fibroblasts fixed in Bouin's fluid and extracted in warm pyridine according to Baker (1946) for 24 hours. Portions of the cytoplasm have apparently been formed into ridges (R and Rd), and loss of identifiable features has occurred both in the nucleus and in the cytoplasm. The cells had been grown on BP in vitro prior to fixation. DMP 1317X

Figure 200. The same cells as in Figure 199 showing the presence of unextracted BP throughout the cytoplasm, in intranuclear structures (N) and associated with the nuclear margin (R). UVFM 1317X

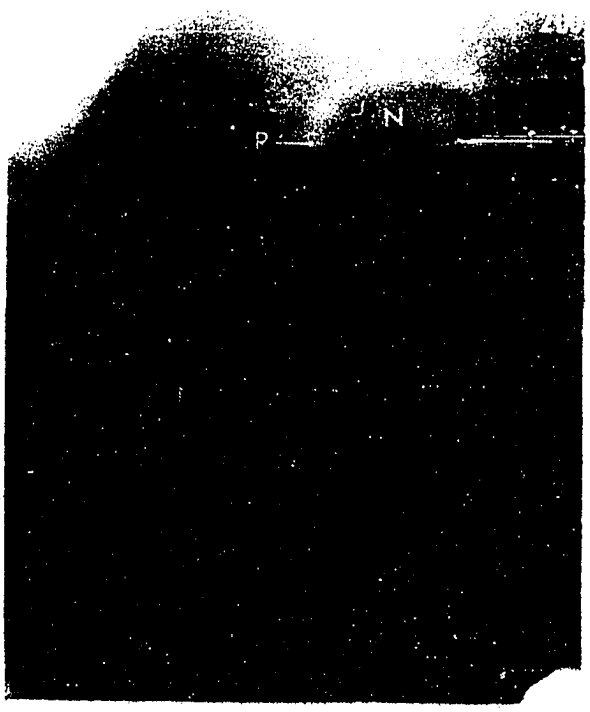
Figure 201. A Bouin's-fixed L-929 fibroblast extracted for 24 hours with warm pyridine; the cell had not been exposed to BP in vitro. U-V-fluorescence photomicrographs showed no evidence of any intracellular fluorescence. Fibrous material is seen in the cytoplasm (F), and the fine cytoplasmic processes (Fp) are preserved. Within the nucleus (N), a structure resembling a nucleolus is evident. DMP 1317X

Figure 202. A Bouin's-fixed L-929 fibroblast extracted for 24 hours with warm pyridine; the preparation to which this cell belongs had not been exposed to BP. No intracellular fluorescence was observed in u-v-fluorescence photomicrographs of the cell. Cytoplasmic ridges (Rd) are seen extending out into a cell process. Within the nucleus (N), a large nucleolus is apparent. DMP 1317X

Figure 203. Bouin's fluid-fixed fibroblasts extracted for 48 hours in warm pyridine; the cells had not been exposed to BP in vitro.

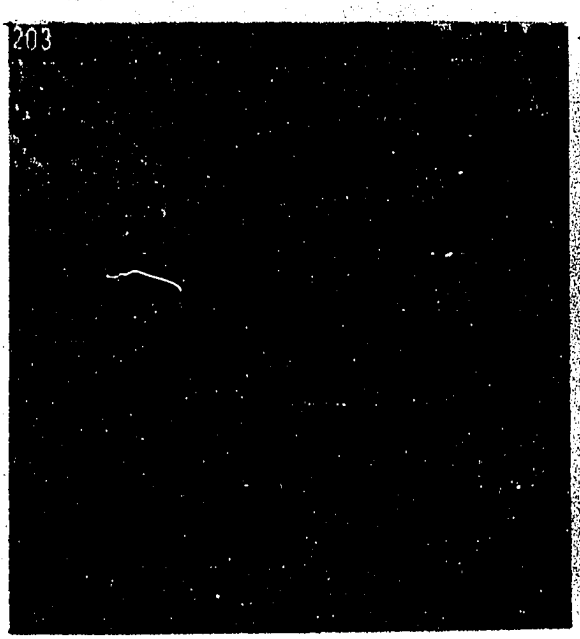
The peripheral cytoplasm is filled with finely precipitated material. Fine cytoplasmic processes are intact (Fp). The nuclear membrane (Nm) is not continuous around the nuclear margin. DMP 1317X

Figure 204. The same cells as in Figure 203 showing a diffuse autofluorescence throughout the cells. UVFM 1317X


P
N


201
Fp


202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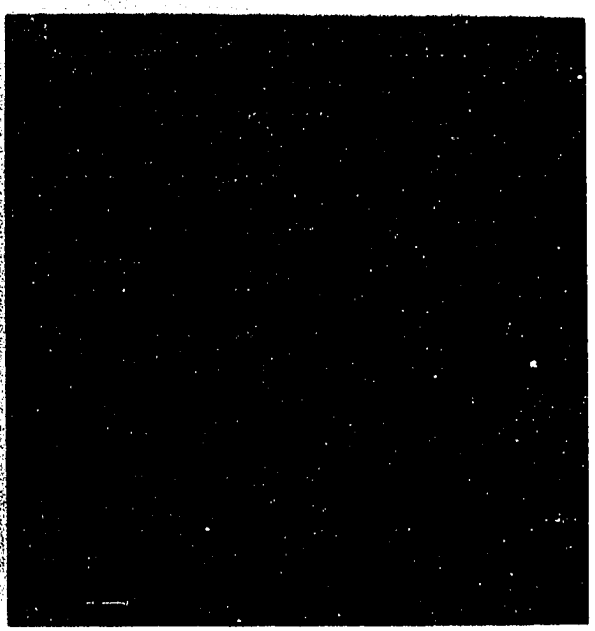

Plate XXVI

Figure 205. A group of Bouin's fluid-fixed fibroblasts treated with Berg's solution. Nucleoli (nc) and the nuclear membranes (Nm) are shown. DMP 1317X

Figure 206. The same cells as in Figure 205 showing a differential BP uptake from Berg's solution in the cytoplasm of the Bouin's fluid-fixed cells. Apparently higher BP accumulation is seen in the region of the nuclear rim (Nm). Demonstrable BP uptake is seen in some nucleoli (nc). UVFM 1317X

Figure 207. A single fibroblast fixed in Bouin's fluid, extracted with $CHCl_3$:MeOH for 5 hours and tested with Berg's solution; the cell was part of a preparation which had not been exposed to BP in vitro and which was non-fluorescence prior to exposure to Berg's BP solution. The nuclear membrane shows numerous breaks. A heavy precipitate of karyoplasm fills the intranuclear region, but a nucleolus is visible (nc). DMP 1317X

Figure 208. The same cell as in Figure 207 showing BP uptake throughout the cytoplasm and by some intranuclear sites, including the nucleolus (nc). UVFM 1317X

Figure 209. A single fibroblast fixed in Bouin's fluid, extracted with $CHCl_3$:MeOH for 5 hours and treated with Berg's BP solution; the cell is part of a preparation which had been grown on BP in vitro. The nuclear membrane shows apparent disjunction at its upper pole. DMP 1317X

Figure 210. The same cell as in Figure 209 showing BP uptake

throughout the cytoplasm and by various intranuclear sites, including apparent nucleoli (nc). UVFM 1317X

Figure 211. Portions of a large fibroblast fixed in $CaCl_2$-HCHO, extracted with $CHCl_3$:MeOH for 5 hours and treated with Berg's solution; the cells are part of a preparation which had not been exposed to BP in vitro. No visible fluorescence was discerned prior to the treatment with Berg's solution. DMP 1317X

Figure 212. The same cell as in Figure 211 showing a general cytoplasmic accumulation of BP from Berg's solution and intranuclear uptake which appears localized in nucleoli (nc). UVFM 1317X

Figure 213. A binucleate fibroblast fixed in $CaCl_2$-HCHO and extracted for 5 hours with $CHCl_3$:MeOH before treatment with Berg's solution; the cell is a portion of a preparation grown on BP in vitro. The preparation was non-fluorescent prior to treatment with the caffeine-BP solution. Well-preserved nucleoli are seen in both nuclei (N). DMP 1317X

Figure 214. The same binucleate cell as in Figure 213 showing a general accumulation of BP by the cytoplasm but no apparent BP uptake by any nuclear structure (N). UVFM 1317X

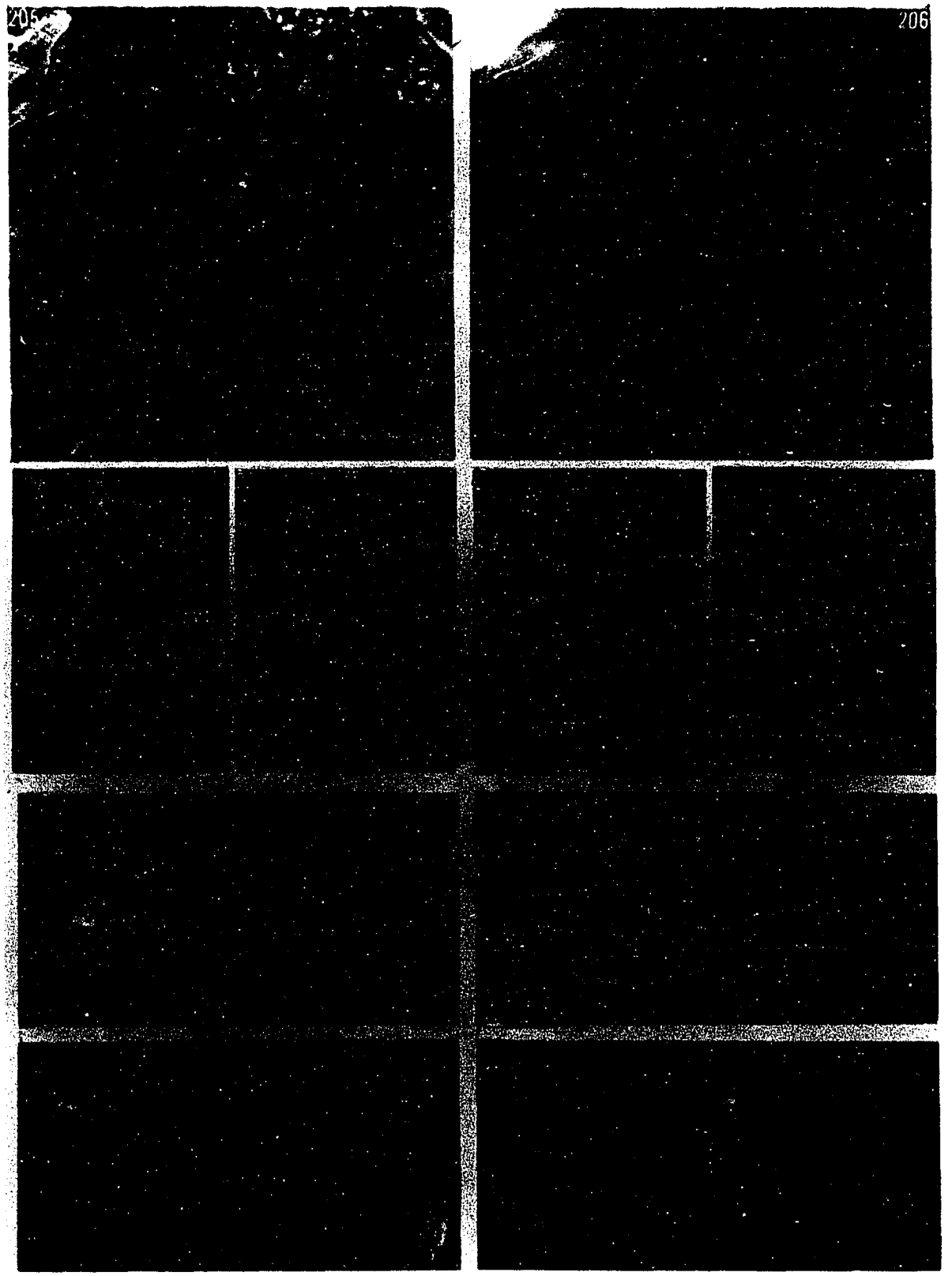
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Plate XXVII

Figure 215. Portions of several cells fixed and extracted in $CHCl_3$:MeOH for 12 hours and treated with Berg's BP; the cells had not been exposed to BP prior to fixation. The cell nuclei (N) are not delimited by any discernible structure. DMP 1317X

Figure 216. The same cells as in Figure 215 showing the general cytoplasmic uptake of BP from Berg's solution. Slightly lower levels of BP uptake are apparent in the general nuclear region (N), compared with the general cytoplasmic uptake. UVFM 1317X

Figure 217. A single L-929 fibroblast fixed and extracted in $CHCl_3$:MeOH for 12 hours followed by treatment with Berg's BP solution; the cells had been grown on crystals of BP in vitro. The nuclear membrane (Nm) is intact except for a segment along the border of the inferior nuclear pole. DMP 1317X

Figure 218. The same cell as in Figure 217 showing a general cytoplasmic uptake of BP and accumulation of BP by intranuclear sites which include nucleoli (nc). UVFM 1317X

Figure 219. A single L-929 fibroblast fixed in EtOH, extracted with $CHCl_3$:MeOH for 12 hours and treated with Berg's caffeine-BP solution; the cell is part of a preparation which had been grown on BP in vitro. Prior to treatment with Berg's solution, however, the cells of the preparation were non-fluorescence due to BP extraction with the lipid solvent. The nuclear membrane appears to be intact in all regions of the nuclear perimeter. DMP 1317X

Figure 220. The same cell as in Figure 219 showing a general accumulation of the CH in the cytoplasmic region. BP uptake by intranuclear structures appears to involve the nucleoli (nc). UVFM 1317X

Figure 221. Several L-fibroblasts, including one in metaphase, which are part of a preparation that was fixed in EtOH, extracted with $CHCl_3$:MeOH for 12 hours and treated with Berg's BP solution; the cells had <u>not</u> been exposed to BP prior to the treatment with Berg's solution. The nuclear membrane surrounding the interphase nucleus is disrupted in many places. Metaphase chromosomal material blends imperceptibly with the cytoplasmic precipitate so that discrete structures on the equatorial plate are not apparent. DMP 1317X

Figure 222. The same cells as in Figure 221 showing a differential cytoplasmic uptake of BP in both the mitotic and interphase cells. No apparent BP uptake is seen in the metaphase chromosomes (C) or within the interphase nucleus (N). UVFM 1317X

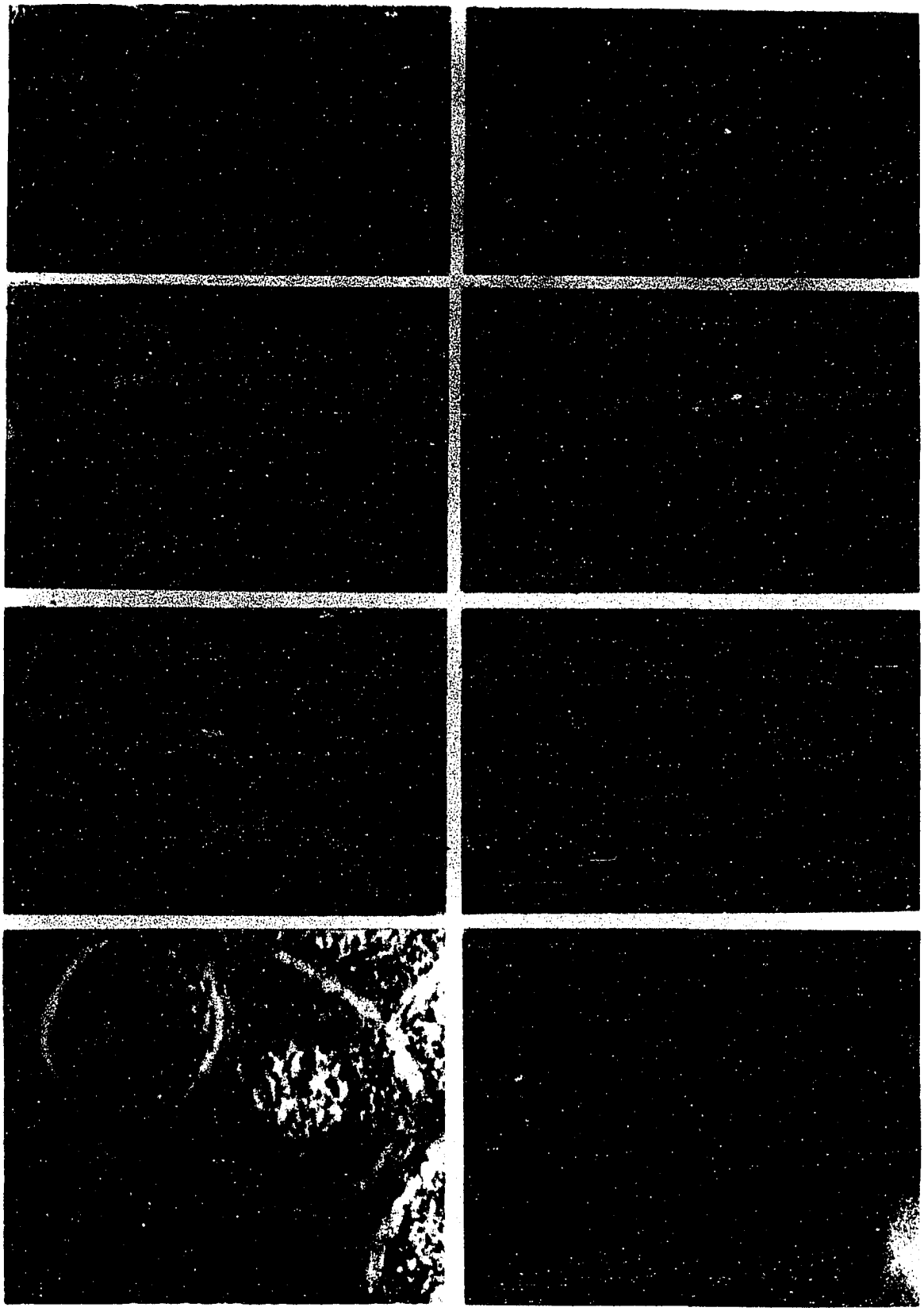

Plate XXVIII

Figure 223. A large mononuclear fibroblast fixed in $CaCl_2$-HCHO and treated with Berg's BP solution. A large Golgi zone (Gz) occurs partly within a concavity in the nucleus. The nuclear margin is well-defined except in the concavity. Nucleoli are distinct structures. The non-nucleolar karyoplasm (Ky) contains no well-defined chromatin structures. DMP 1317X

Figure 224. The same cell as in Figure 223 showing the relatively greatest accumulation of BP from Berg's solution in the Golgi zone (Gz). All the nuclear structures show no comparative evidence of any BP accumulation (N). Fluorescence associated with the nuclear region apparently originates from sub- or supranuclear cytoplasm. UVFM 1317X

Figure 225. A spindle-shaped L-929 fibroblast fixed in $CaCl_2$-HCHO, dehydrated in EtOH, cleared in toluene, rehydrated and treated with Berg's caffeine-BP solution. Nuclear structures such as nucleoli (nc) are well preserved. DMP 1317X

Figure 226. The same cell as in Figure 225 showing a generally faint cytoplasmic uptake of BP and an intranuclear accumulation of CH which is apparently localized in the nucleolus (nc). UVFM 1317X

Figure 227. Three L-929 fibroblasts from a preparation fixed in EtOH and treated with Berg's caffeine-BP solution. Within the precipitated cytoplasm and nucleoplasm, a Golgi zone (Gz) and nucleoli (nc) are discernible. DMP 1317X

Figure 228. The same cells as in Figure 227 showing a barely differential uptake of BP by the cytoplasm. Slightly higher Bp accumulations are seen in the general Golgi zone (Gz). Evidence of slight BP uptake by nucleoli (nc) also is visible. UVFM 1317X

Figure 229. A stellate fibroblast fixed in complete fresh Zenker's fluid followed by post-treatment with Lugol's solution and thiosulfate to remove mercury and by treatment with Berg's caffeine-BP solution. A Golgi zone (Gz) is demonstrable in the cytoplasm. The nucleus is generally highly refractile (N), but is discernibly surrounded by an intact nuclear membrane. DMP 1317X

Figure 230. The same cell as in Figure 229 showing relatively highest accumulations of BP in the cytoplasm situated in the Golgi zone (Gz) of the fibroblast. No apparent BP uptake by any nuclear structure is demonstrable. UVFM 1317X

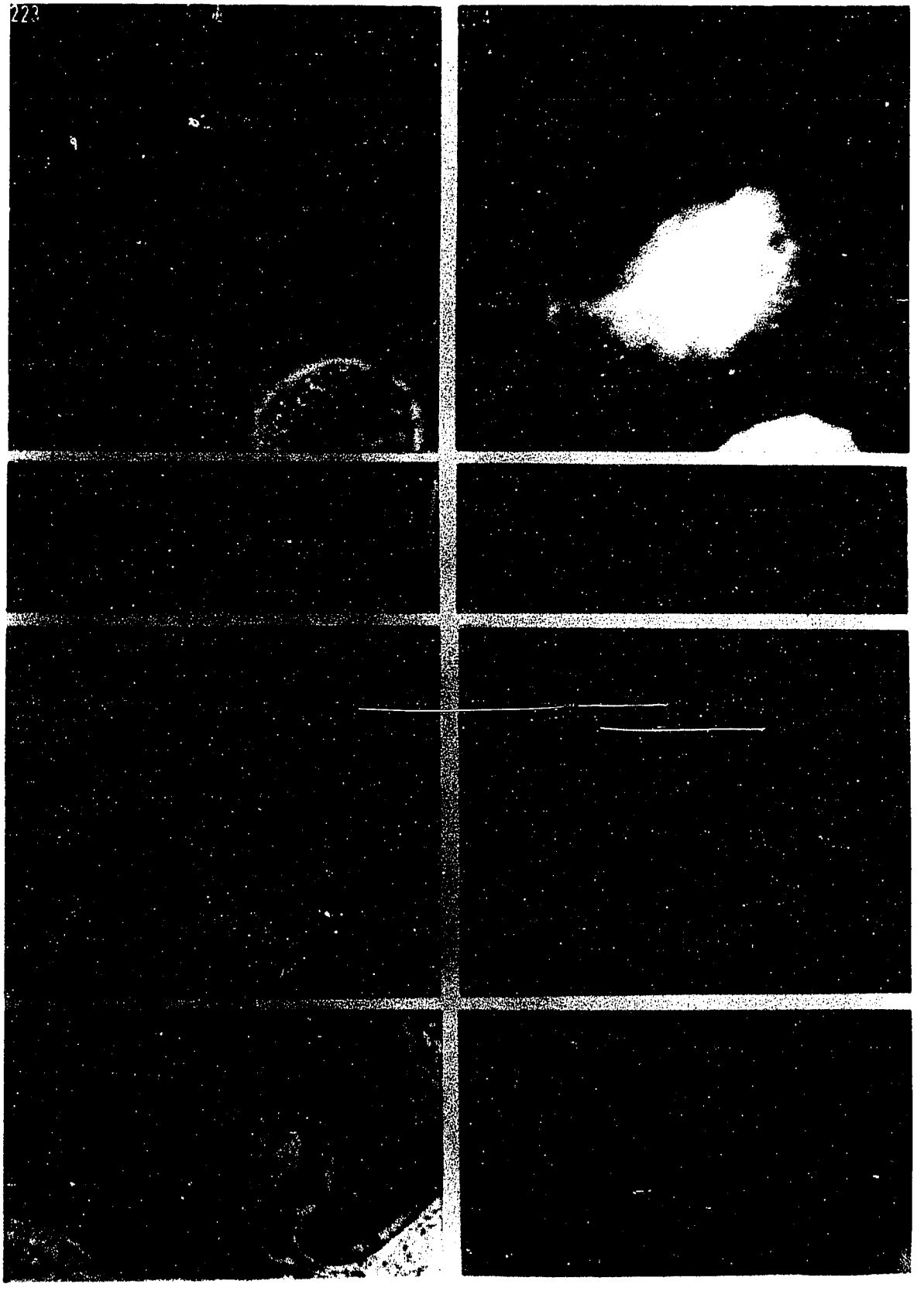
223

Plate XXIX

Figure 231. A group of three hepatic parenchymal cells showing a possible Golgi complex (Gz), lipid droplets (L) and the small granule component (Sg). The cells' nuclei are surrounded by distinct nuclear membranes (Nm) and contain one to several nucleoli and visible chromatin material (H). DMP 1317X

Figure 232. The same group of cells as in Figure 231 showing the highest relative cytoplasmic uptake localized in the lipid droplets (L) and the small granule component (Sg). Apparent BP uptake by the Golgi complex (Gz) is greater than that of the general cytoplasmic matrix. No accumulation of the CH by any nuclear structure is evident (N). UVFM 1317X

Figure 233. An hepatic cell is seen with a single nucleus containing chromatin (H) and nucleoli (nc). In the cytoplasm, filamentous mitochondria (M), a Golgi complex (Gz) and frank lipid droplets (L) are discernible. DMP 1317X

Figure 234. The same cell as in Figure 233 showing relatively high accumulation of BP in the region of the Golgi complex (Gz) and in lipid droplets (L). No localization of CH is displayed in mitochondria (M) or in any nuclear structure (N). UVFM 1317X

Figure 235. Portions of several hepatic cells, all of which are ruptured. A vesicular Golgi zone (Gz) is seen amid the granular mitochondria. The nuclei show several nucleoli and distinct chromatin material (H). An isolated nucleus appears in the upper right side of the photograph. DMP 1317X

Figure 236. The same cells as in Figure 235 showing demonstrable accumulations of BP in the Golgi complex (Gz) and no apparent uptake of BP by any nuclear structure. UVFM 1317X

Figure 237. A large binucleate hepatocyte with a number of presumptive Golgi complexes (Gz), filamentous mitochondria (M) and the small granule component (Sg). DMP 1317X

Figure 238. The same binucleate cell as in Figure 237 showing relatively higher BP accumulation in the Golgi complex (Gz) and the small granule component (Sg). No demonstrable accumulation of BP occurs in mitochondria (M) or any nuclear structure. UVFM 1317X

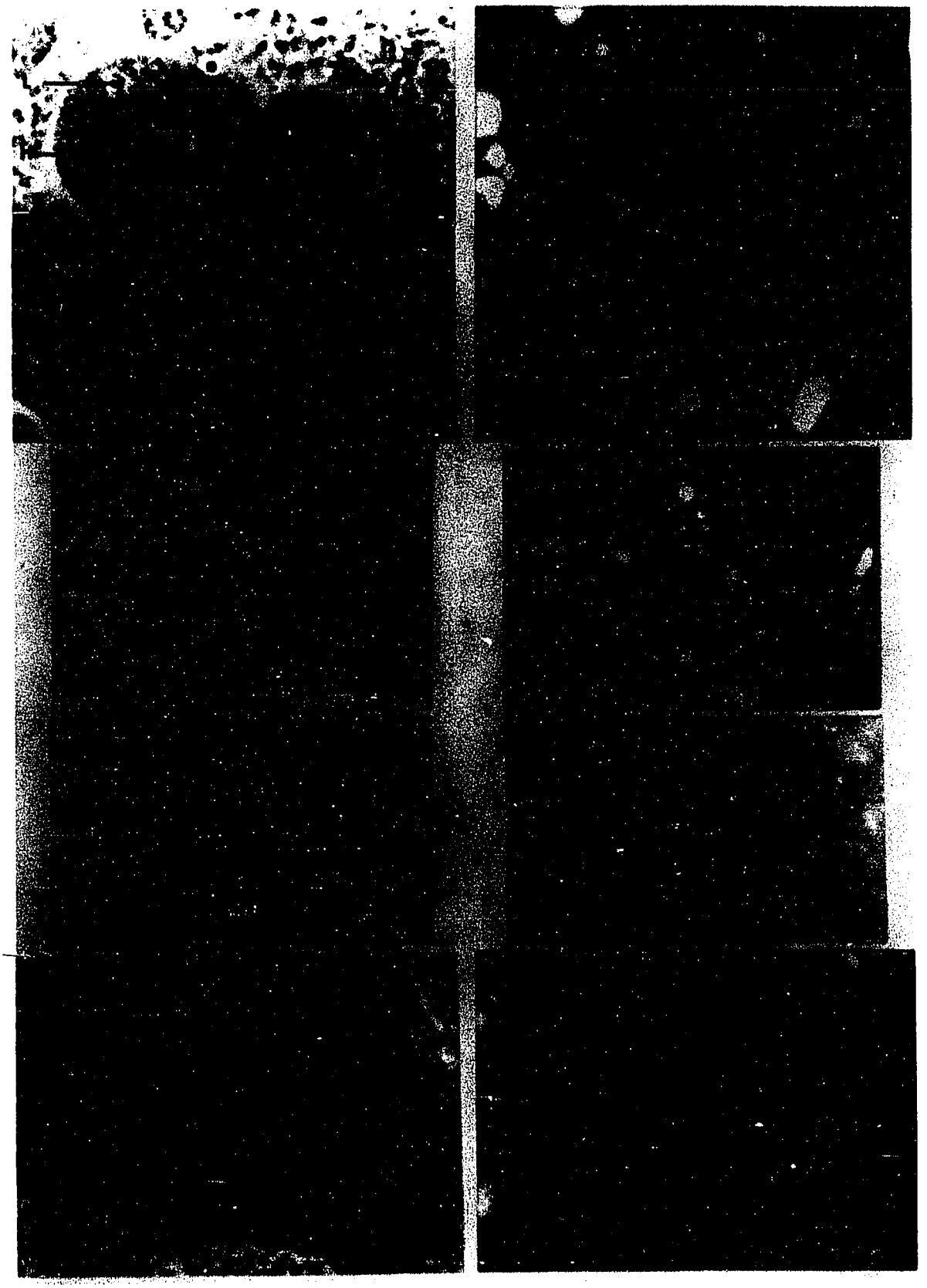

Plate XXX

Figure 239. A portion of a 10 micra paraffin section of ultracentrifuged liver stained conventionally with Hematoxylin (Lillie-Meyer) and Eosin. Most of the basophilic material of the nucleus (H) is seen sedimented at the centrifugal nuclear pole. Different strata are indicated by numbers. See the text (Chapter VIII) for an explanation. OLM 1317X

Figure 240. Paraffin section of ultracentrifuged liver stained with Gallocyanin-Chrom Alum after treatment with RNAase. DNA is thus shown to occur in the sedimented mass at the centrifugal end of the nucleus, in the entire margin of the nucleus and as threads stretching between the centrifugal and centripetal nuclear poles (H). OLM 1317X

Figure 241. Paraffin sections of ultracentrifuged liver stained with Gallocyanin-Chrom Alum after hydrolysis in DNAase. The demonstrable RNA within the nucleus (H) is seen to occupy the same apparent sites as the DNA demonstrated in Figure 240. Cytoplasmic RNA is seen to occur in the 3rd and 4th strata but not in the more centripetal 2nd stratum. OLM 1317X

Figure 242. Paraffin sections of ultracentrifuged liver stained with Feulgen reaction. The sites of DNA are seen to be the same as those demonstrated in Figure 240. DNA (H) appears in the centrifugal nuclear mass, the marginal rim and in threads connecting centripetal and centrifugal nuclear poles. OLM 1317X

Figure 243. A paraffin section of ultracentrifuged liver stained with Gallocyanin-Chrom Alum without prior enzymatic treatment. The centripetal portion of the nucleus, excluding chromatin threads and margin, is apparently free of nucleic acid as is the 2nd cytoplasmic stratum. OLM 1317X

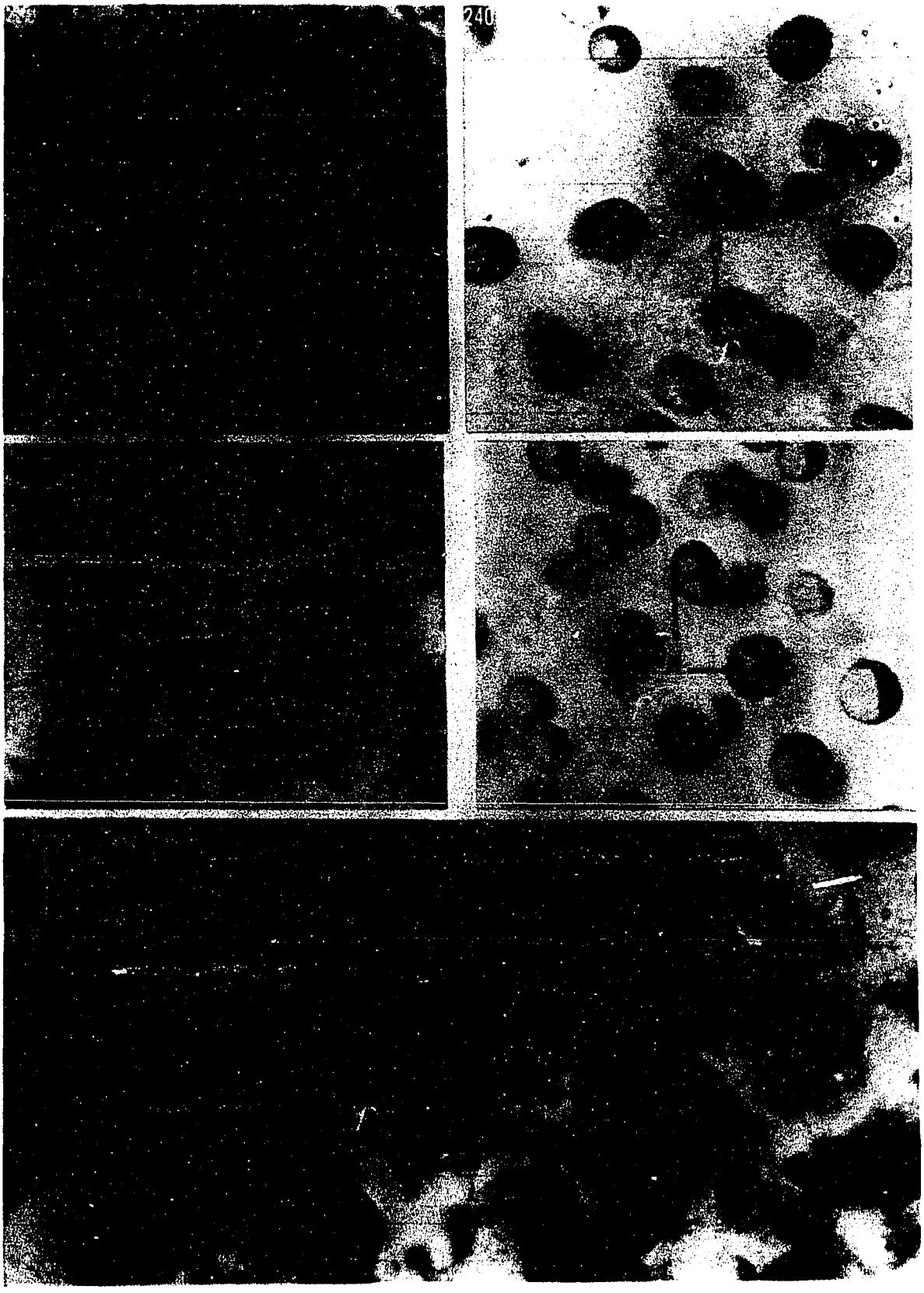
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Plate XXXI

Figure 244. Several dissociated hepatic parenchymal cell after ultracentrifugation at 400,000 x G for 2 hours. Cytoplasmic strata #1 and #3 are evident, while no apparent 2nd stratum is seen, and the 4th stratum is indistinct. The nuclear rim (R) is a well-defined structure surrounding the stratified nucleus, most of whose chromatin has been sedimented toward the centrifugal pole (to the left). Some members of the small granule component (Sg) are visible among the lipid droplets of the 1st stratum. DMP 1317X

Figure 245. The same cells as in Figure 244 showing the relatively highest BP accumulations in the lipid droplets of the first stratum (L) and in the members of the small granule component (Sg). No BP uptake is discernible anywhere in the centrifuged nucleus (N). UVFM 1317X

Figure 246. Hepatic parenchymal cells after ultracentrifugation at 400,000 x G for 2 hours. The central cell has been stratified into four strata (#2, #3, and #4) although the 1st stratum at the centripetal (upper) end of the cell is indiscrete. Golgi zones appear (Gz) in both the 2nd and 3rd strata, and vesicular mitochondria are seen in the 3rd stratum (M). Vacuolar structures (Va) occur in the 2nd stratum. The cell nucleus of the central cell shows large amounts of centrifugally displaced chromatin (H) and some thin chromatic threads running up to the centripetal portions of the nucleus. DMP 1317X

Figure 247. The same cells as in Figure 246 showing relatively

higher BP uptake in portions of the small granule component (Sg) and in the Golgi complex (Gz). The centrifugal edge (lower surface of the central cell)(marked "4") also shows a relatively higher BP accumulation. No apparent BP uptake occurs in the mitochondria (M) or in any nuclear structure (N) or in cytoplasmic vacuolar elements (Va). A relatively lower BP concentration is apparent in the region of the 2nd cytoplasmic stratum. UVFM 1317X

Figure 248. A pair of binucleate rat hepatic cells after ultracentrifugation at 400,000 x G for 2 hours in physiologic medium. Numbers indicate the various intracellular strata. Mitochondria (M), a Golgi zone (Gz) and elements of the small granule component (Sg) are discernible. Nuclear chromatin is stratified against the centrifugal pole of the nucleus (H). DMP 1317X

Figure 249. The same pair of cells as in Figure 248 showing relatively higher BP accumulation in the Golgi zones (Gz) and elements of the small granule component (Sg) are demonstrable. BP concentration in the 2nd stratum (2) is apparently lower than that in the general cytoplasmic matrix. No evident BP accumulation is seen in any nuclear structure (N) or in mitochondria (M). UVFM 1317X

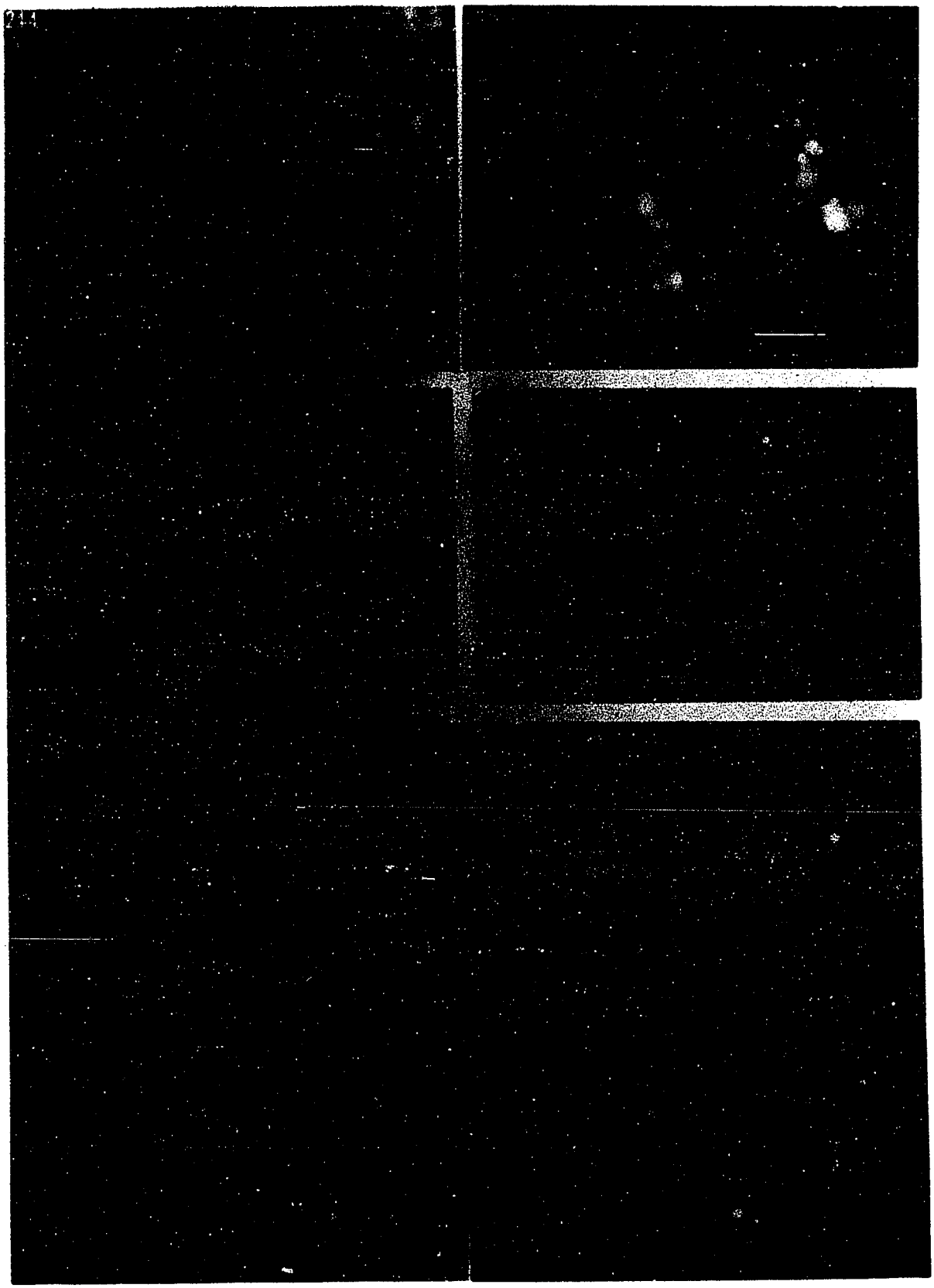
244

Plate XXXII

Figure 250. Group of nuclei isolated in 0.25M sucrose-$CaCl_2$ showing a dark nuclear rim (R), nucleoli and nearly homogenous non-nucleolar karyoplasm (Ky). Cytoplasmic debris fills intervals between nuclei. DMP 1317X

Figure 251. Same group of nuclei as in Figure 250 showing BP uptake on the nuclear rim (F), but not within the nuclear internum (N). UVFM 1317X

Figure 252. Group of nuclei isolated in 0.25M sucrose-$CaCl_2$ showing dark nuclear rims (F), nucleoli (nc) and a slightly heterogeneous non-nucleolar karyoplasm. The nuclei are situated within a loose mass of protoplasmic debris. DMP 1317X

Figure 253. Same group of nuclei as in Figure 252 showing no BP uptake by any nuclear structure within the confines of the nuclear rim (F), which itself shows a relatively high BP uptake. UVFM 1317X

Figure 254. Group of nuclei isolated in 0.25M sucrose without $CaCl_2$. The karyoplasm of the nucleus is hyaline (Ky) and uninterrupted by discernible structures. A nuclear rim surrounds the clear hyaline sphere. DMP 1317X

Figure 255. Same nucleus as in Figure 254 showing that the only demonstrable uptake of BP by the nucleus is located in the nuclear rim (R). UVFM 1317X

Figure 256. Group of agglutinated nuclei isolated in 0.25M sucrose-$CaCl_2$ and extracted with $CHCl_3$:MeOH. The nuclei are filled with a coarse non-nucleolar karyoplasmic precipitate, one to several

nucleoli and are circumscribed by a refractile rim (R). A mass of debris occupies the upper center of the photomicrograph. DMP 1317X

Figure 257. Same group of nuclei as in Figure 256 showing the shadow of a nuclear rim (R) overlying a BP crystal. The preparation has been treated with Berg's BP and also exposed to crystalline BP. No demonstrable uptake of BP is notable except in the central debris. UVFM 1317X

Figure 258. Three intact nuclei and one broken nucleus situated on the edge of a clot of debris. Distinct nucleoli (nc), nuclear rims (R) and slightly heterogeneous non-nucleolar karyoplasm are discernible in this 0.88M sucrose-$CaCl_2$ preparation. DMP 1317X

Figure 259. Same nuclei as in Figure 258 showing no demonstrable BP accumulation anywhere in the nuclei (N), except in the nuclear rim (R). UVFM 1317X

Figure 260. Group of nuclei isolated in 0.88M sucrose without $CaCl_2$ showing a hyaline, undifferentiated karyoplasm (Ky) surrounded by a nuclear rim (R). On the right side of the picture, a broken nucleus is visible. DMP 1317X

Figure 261. Same group of nuclei as in Figure 260 showing no nuclear accumulation of BP (N), except by the nuclear rim (R). The broken nucleus on the right side of the picture shows the same pattern.

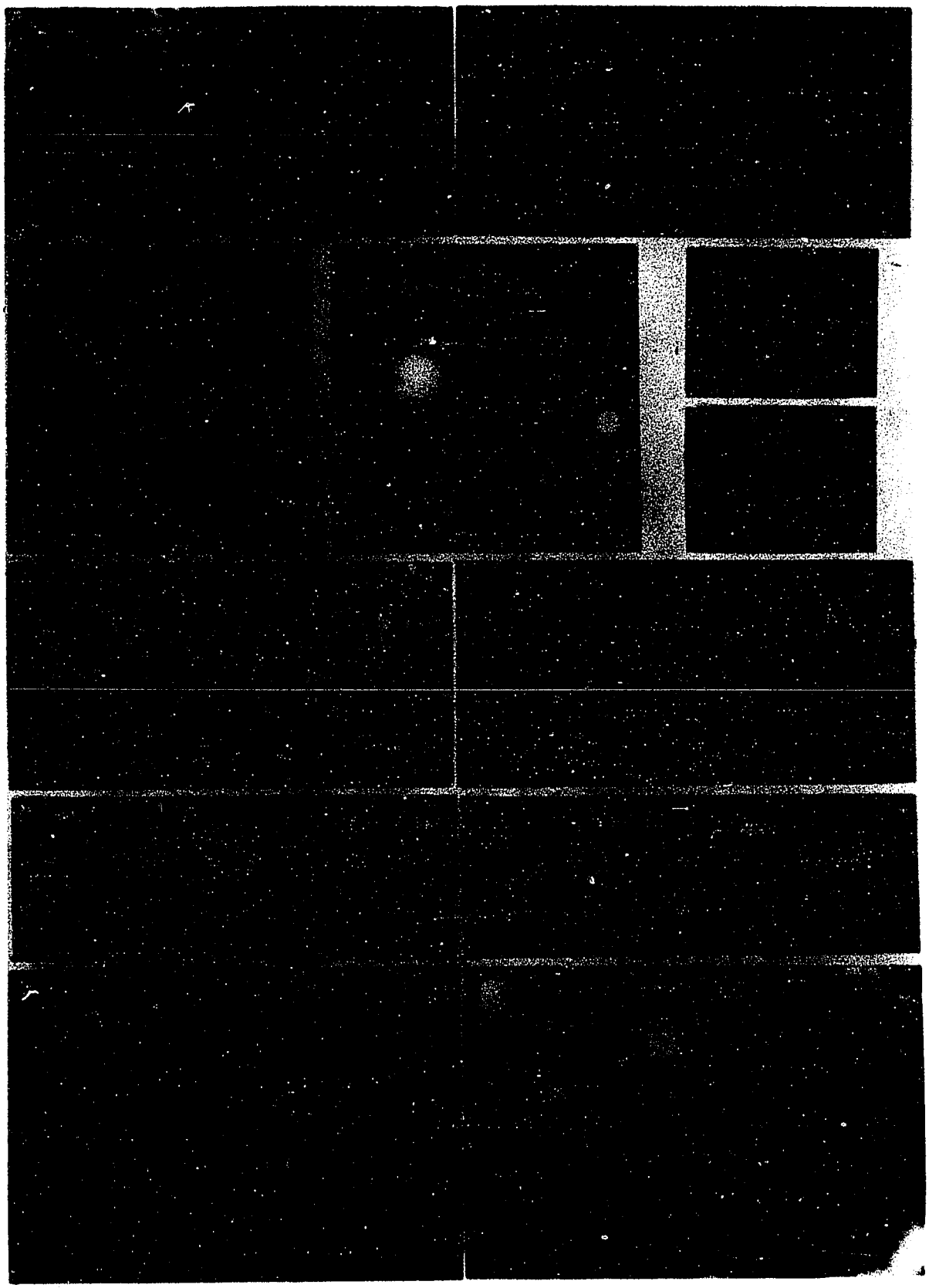

Plate XXXIII

Figure 262. A liver nucleus isolated in 2.2M sucrose, showing a nucleolus just out of focus (nc), a clear non-nucleolar karyoplasm and a discrete nuclear rim (R). DMP 1317X

Figure 263. Same liver nucleus as in Figure 262 showing uptake of BP only in the nuclear rim (R). UVFM 1317X

Figure 264. A liver nucleus isolated by Dounce's pH 3.8 citric acid procedure. Several small nucleoli amid a slightly precipitated non-nucleolar karyoplasm (Ky) are distinct. The nuclear rim (R) is somewhat obscured by its refractility. DMP. 1317X

Figure 265. Same cells as in Figure 264 showing a diffuse BP accumulation by the general karyoplasmic region of the nucleus (Ky) and a higher relative accumulation in the nuclear rim (R). UVFM 1317X

Figure 266. A nucleus isolated using Dounce's pH 6.0 citric acid procedure showing a coarsely differentiated karyoplasm (Ky) and a refractile nuclear rim (R). DMP 1317X

Figure 267. Same cell as in Figure 266 showing a general uptake of BP by the karyoplasm, localized in slightly higher concentrations by the precipitated karyoplasmic material and by the nuclear rim. UVFM 1317X

Figure 268. A liver nucleus isolated by Dounce's pH 3.8 weak citric acid procedure. A single nucleolus is situated in a coarsely precipitated karyoplasm (Ky). The nuclear rim (R) is refractile. DMP 1317X

Figure 269. Same nucleus as in Figure 268 showing general BP uptake throughout the entire karyoplasm (Ky) with local slightly higher accumulations in the precipitated karyoplasmic material and the nuclear rim (R). UVFM 1317X

Figure 270. A single nucleus isolated in 1% citric acid, distorted in shape, filled with a coarsely precipitated karyoplasm (Ky) and highly refractile. DMP 1317X

Figure 271. Same nucleus as in Figure 270 showing a BP uptake by the general karyoplasmic region and slightly higher accumulations in specific karyoplasmic masses and in the nuclear rim. UVFM 1317X

Figure 272. A shrunken, distorted liver nucleus as isolated in 1% citric acid, showing a highly precipitated karyoplasm (Ky). DMP 1317X

Figure 273. Same nucleus as in Figure 272 showing a diffuse BP uptake throughout the entire nucleus and its rim. UVFM 1317X

Figure 274. A group of liver nuclei isolated in 1M citric acid. Distortion in nuclear shape is apparent. Nucleoli (nc) are discernible within the otherwise precipitated karyoplasm (Ky). Nuclear rims (R) are somewhat obscured by the nuclear refractility. DMP 1317X

Figure 275. Same group of nuclei as in Figure 274 showing distinct accumulations of BP in nucleoli (nc) and other karyoplasmic precipitated masses (ky). Nuclear rims (R) also display a relatively high BP uptake. UVFM 1317X

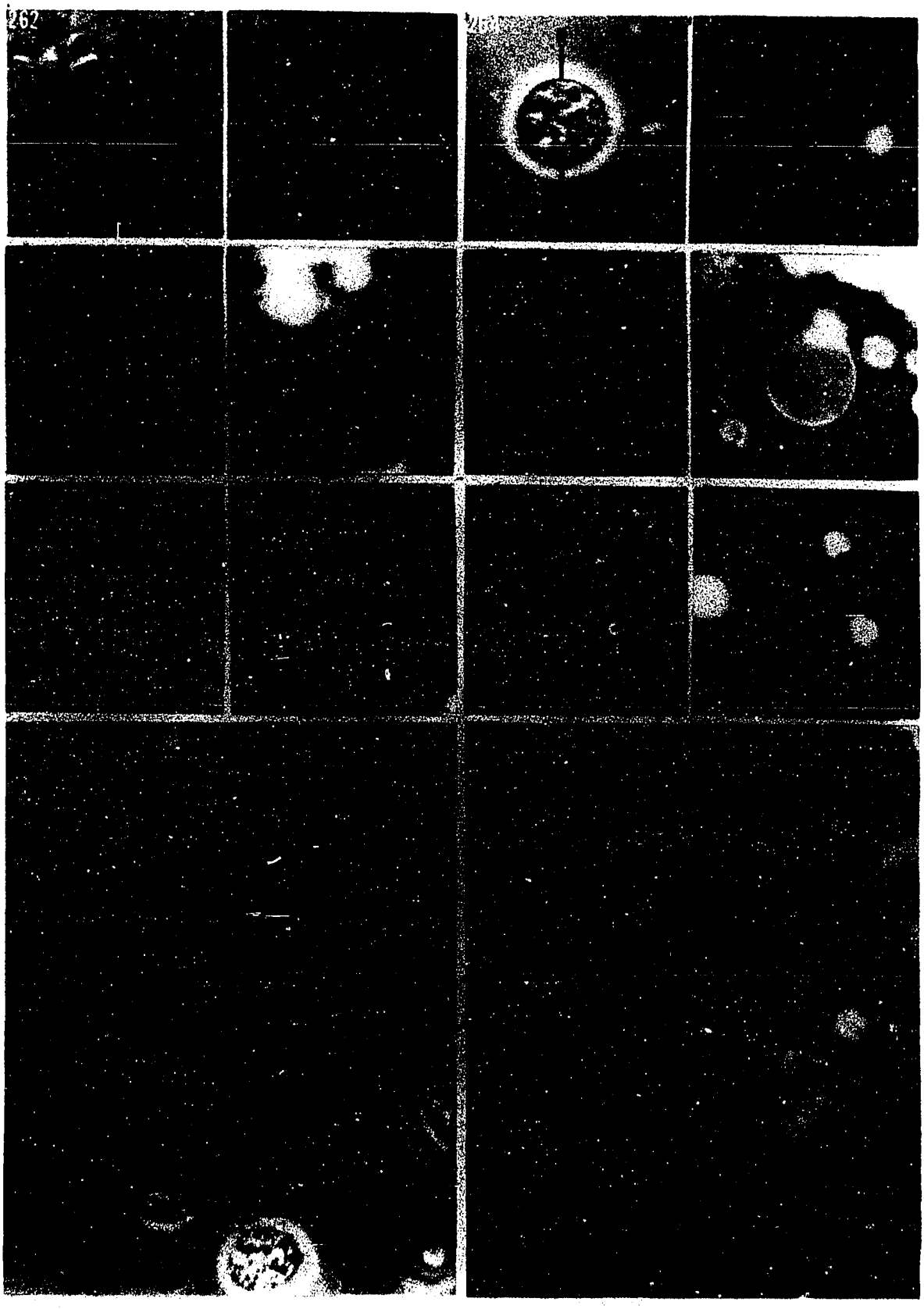
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Plate XXXIV

Figure 276. Two nuclei isolated in 0.25M sucrose $CaCl_2$ and resuspended in McIlvaine's buffer, pH 4.8. A single dark, refractile nucleolus (nc) is seen in the center nucleus. The non-nucleolar karyoplasm is generally dark and coarsely clumped. The nuclear rim (R) is somewhat obscured by the interference patterns at the nuclear edge. DMP 1317X

Figure 277. Same nucleus as in Figure 276 showing that the only BP uptake by the nucleus is localized in the nuclear rim (R) and cytoplasmic tags adherent to it. UVFM 1317X

Figure 278. Same nucleus as in Figures 276 and 277 showing a slightly higher focal plane. Nucleolar-associated chromatin material can be seen surrounding the nucleolus (nc). DMP 1317X

Figure 279. Group of nuclei isolated in 0.25M sucrose-$CaCl_2$ and resuspended in McIlvaine's buffer at pH 4.8. The nuclei are associated with much debris. They contain one to several nucleoli (nc), a dark, coarsely clumped non-nucleolar karyoplasm (Ky) and a dark indistinct nuclear rim. DMP 1317X

Figure 280. The same group of nuclei as in Figure 279 showing that BP uptake by the nuclei is confined to the nuclear rim (R). UVFM 1317X

Figure 281. A single nucleus isolated in 0.25M sucrose-$CaCl_2$ and resuspended in McIlvaine's buffer, pH 6.0. The dark non-nucleolar karoplasm is nearly homogeneous (Ky), contains a single nucleolus and is surrounded by a discrete nuclear rim (R). DMP 1317X

Figure 282. Same nucleus as in Figure 281 showing BP uptake confined to the nuclear rim (R). UVFM 1317X

Figure 283. A single nucleus suspended in pH 6.0 McIlvaine's buffer. The dark, nearly homogeneous karyoplasm contains two dark nucleoli and an indistinct nuclear rim (R). DMP 1317X

Figure 284. The same nucleus as was pictured in Figure 283. The CH is apparently localized only in the nuclear rim (R). UVFM 1317X

Figure 285. A pair of nuclei with adherent cytoplasmic debris isolated in 0.25M sucrose-$CaCl_2$ and suspended in acetate buffer, pH 6.0. Nucleoli (nc) are situated in a finely precipitated karyoplasm (Ky) which also contains a few clear spaces. The nuclear rim (R) is obscured by interference rings from the refractile nucleus. DMP 1317X

Figure 286. The same nuclei as in Figure 285. BP uptake is greatest in the nuclear rim (R), but the suggestion of some BP diffusely accumulated by the general karyoplasm (Ky) can be seen. UVFM 1317X

Figure 287. A single nucleus at the edge of some debris in pH 4.9 acetate buffer. One to two small dark nucleoli are located in a coarsely precipitated karyoplasm. The whole nucleus is highly refractile so that the nuclear rim (R) is obscured. DMP 1317X

Figure 288. Same nucleus as in Figure 287 showing karyoplasmic (Ky) uptake of BP, without noticeable localization in any intranuclear structure. Highest apparent BP uptake occurs in the nuclear rim (R). UVFM 1317X

Figure 289. The same nucleus as in Figure 288 showing some wrinkling in nuclear rim (R). DMP 1317X

Figure 290. The same cell as in Figures 287-289 at a slightly different focal plane. No discrete localization of BP in any karyoplasmic (Ky) structure can be made out at this level. Highest relative BP uptake occurs in the nuclear rim (R). UVFM 1317X

Figure 291. A group of nuclei suspended in pH 4.9 acetate buffer. The coarsely clumped karyoplasm (Ky) contains several nucleoli. The nuclear rim is obscured by the interference rings of the refractile nuclei. DMP 1317X

Figure 292. Same group of nuclei as in Figure 291. Accumulation of BP by the general karyoplasmic region of all the nuclei (Ky) is seen, and the only differential accumulation occurs in the nuclear rim (R). UVFM 1317X

Figure 293. Same group of nuclei as in Figures 291 and 292. The nucleus in the lower left side of the picture has rotated counterclockwise, exposing a new focal plane containing the same structures as in Figure 291. DMP 1317X

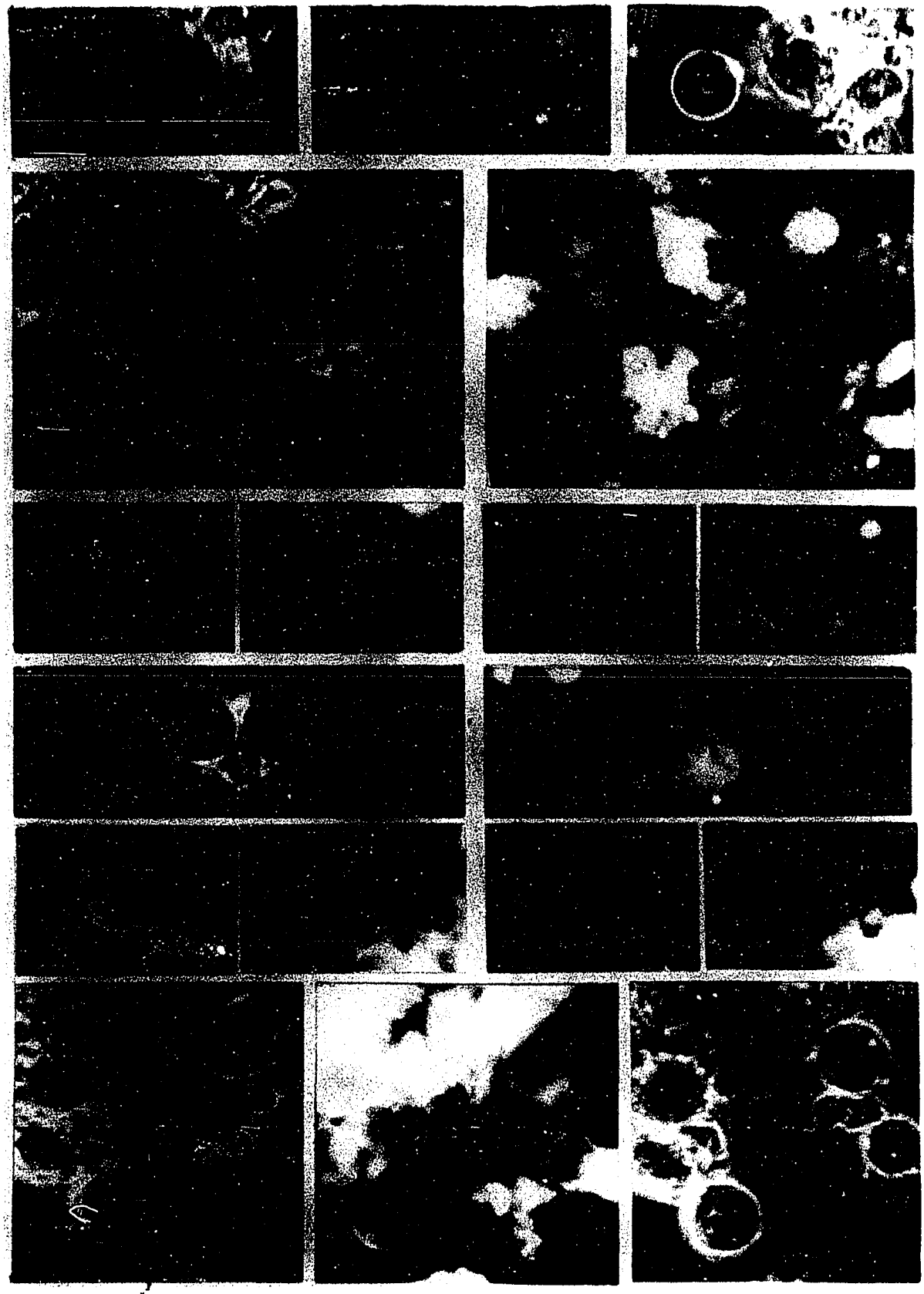


Plate XXXV

Figure 294. Two nuclei isolated in 0.25M sucrose-$CaCl_2$ and resuspended in 0.1M sodium phosphate buffer, pH 5.5. Single nucleoli (nc) are situated in a finely heterogeneous karyoplasmic matrix. The nuclear rim (R) is wrinkled and, in the right hand nucleus, has produced a small bleb (B). DMP 1317X

Figure 295. Same two nuclei as in Figure 294 showing no BP uptake by the general karoplasm of the nuclei (Ky) or by the internum of the nuclear bleb (B). The rims (R) of the nucleus and walls of the bleb show BP uptake. UVFM 1317X

Figure 296. A pair of nuclei suspended in Na-phosphate buffer, pH 5.5, showing one to several nucleoli (nc) in a somewhat coarsely clumped karyoplasm, surrounded by a nuclear rim (R). DMP 1317X

Figure 297. The same pair of nuclei as in Figure 296 showing the confinement of BP uptake to the nuclear rim (R). The appearance of some fluorescence just inside the nuclear rims is due to BP accumulations in parts of the rim just above or below the focal plane. UVFM 1317X

Figure 298. Same pair of nuclei at a different focal plane as shown in Figure 296 and 297. DMP 1317X

Figure 299. Same pair of nuclei as in Figures 296-298. UVFM 1317X

Figure 300. A single nucleus suspended in Na-phosphate buffer, pH 7.4, showing a clear, undifferentiated hyaline karyoplasm (Ky)

surrounded by a thin nuclear rim (R). DMP 1317X

Figure 301. Same nucleus as in Figure 300 showing a BP accumulation by the general karyoplasm (Ky) and a higher BP uptake in the nuclear rim (R). UVFM 1317X

Figure 302. A single nucleus suspended in Na-phosphate buffer, pH 7.4. No nucleoli are discernible in the homogeneous karyoplasm (Ky). A bleb (B) is visible originating from the nuclear rim (R). DMP 1317X

Figure 303. The same nucleus as in Figure 302 showing no apparent BP uptake by any material within the confines of the nuclear rim (R), which shows discernible BP localization (R). The rim of the nuclear bleb (B) also displays BP uptake. UVFM 1317X

Figure 304. A single nucleus suspended in Na-phosphate buffer, pH 5.5. The nucleus, in contrast with those in Figures 294-299, shows a homogeneous non-nucleolar karyoplasm (Ky) and no evidence of wrinkling on the nuclear rim. DMP 1317X

Figure 305. The same nucleus as in Figure 304 showing no BP uptake by karyoplasmic structures and a discrete BP accumulation in the nuclear rim (R). UVFM 1317X

Figure 306. A group of nuclei isolated in 0.25M sucrose-$CaCl_2$ and suspended in K-phosphate buffer, pH 7.4. The nuclei show one to several nucleoli (nc), a homogenous karyoplasm, distinct nuclear rims and several blebs (B). DMP 1317X

Figure 307. Same nuclei as in Figure 306 displaying no BP uptake by karyoplasmic structures or in the bleb interna (B). Both

the nuclear and the bleb rims (R) show high relative BP accumulations. UVFM 1317X

Figure 308. Two nuclei suspended in K-phosphate buffer, pH 7.4. A dark nuclear rim (R) surrounds a clear, undifferentiated non-nucleolar karyoplasm. One to two dark nucleoli are visible (nc). DMP 1317X

Figure 309. Same two nuclei as in Figure 308 showing the only localization of BP relative to the nuclei is in the nuclear rim (R). UVFM 1317X

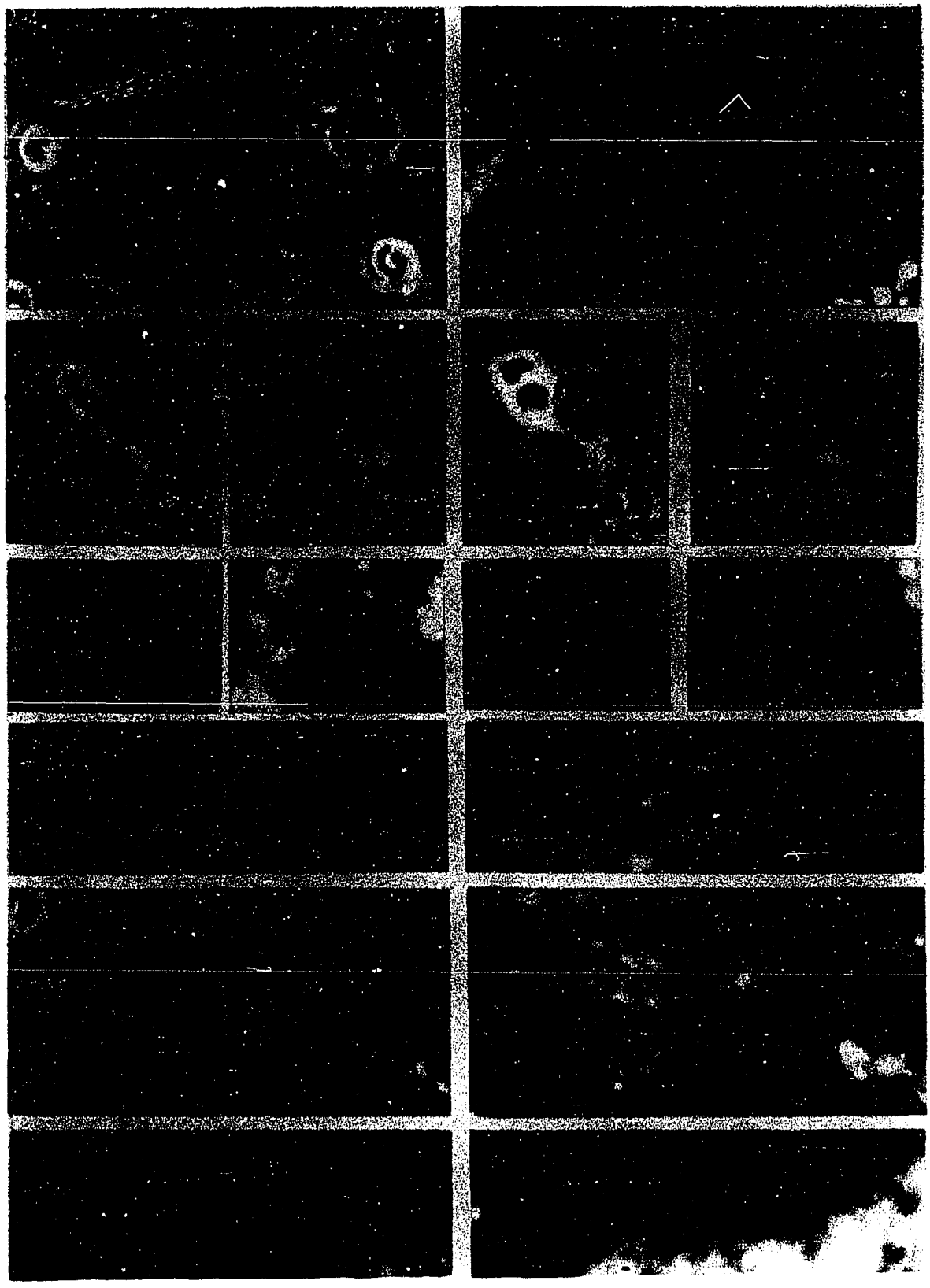


Plate XXXVI

Figure 310. An isolated liver nucleus incubated six hours at 37°C in McIlvaine's buffer without $MgCl_2$. Numerous blebs (B) appear above the nucleus, which contains two nucleoli and is enclosed by a distinct nuclear rim (R). DMP 1317X

Figure 311. The same nucleus as in Figure 310 showing demonstrable BP uptake by the nuclear rim (R) and the walls of the nuclear blebs (B) but not by material on the internum of either the nucleus or the bleb. UVFM 1317X

Figure 312. A nucleus incubated in McIlvaine's buffer for six hours at 37°C in McIlvaine's buffer without $MgCl_2$. Numerous blebs (B) are seen. DMP 1317X

Figure 313. The same nucleus as in Figure 312 showing no apparent BP uptake by any karyoplasmic structure (Ky) or in the internum of the nuclear blebs. (B). UVFM 1317X

Figure 314. An isolated liver nucleus with bleb after incubation in McIlvaine's buffer at 37°C for six hours in the presence of $MgCl_2$. DMP 1317X

Figure 315. The same nucleus as in Figure 314 showing no demonstrable localization of BP in any nuclear structure except the rim (R). UVFM 1317X

Figure 316. An isolated liver nucleus incubated in pH 6.0 McIlvaine's buffer at 37°C for 6 hours not in the presence of $MgCl_2$. The karyoplasm (Ky) shows evidence of heterogeniety. DMP 1317X

Figure 317. The same nucleus as in Figure 316 showing no

evident BP uptake by karyoplasmic structures (Ky). The fluorescence over the center of the nucleus is probably due to BP concentrations on the nuclear rim above/below the plane of focus. UVFM 1317X

Figure 318. An isolated hepatic nucleus incubated in pH 6.0 McIlvaine's buffer at 37°C for six hours in the presence of $MgCl_2$. A nucleolus (nc) and a rim bleb (B) are seen. DMP 1317X

Figure 319. The same nucleus as in Figure 318 showing high BP uptake in the nuclear rim (R). The BP-fluorescence over the interior of the nucleus is interpreted as due to BP concentrations located in structures on the nuclear rim and not within the confines of the rim. UVFM 1317X

Figure 320. An isolated liver nucleus incubated in McIlvaine's buffer at pH 6.0 for 6 hours at 37°C with $MgCl_2$. DMP 1317X

Figure 321. The same nucleus as in Figure 320 showing BP uptake by the nuclear rim (R) and by the walls of the nuclear bleb (B). Fluorescence over the nuclear interior does not appear localized in any specific karoplasmic structure and therefore is thought to arise from structures on the nuclear rim. UVFM 1317X

Figure 322. An isolated liver nucleus with several blebs (B) incubated in the complete DNAase incubation medium. The non-nucleolar karyoplasm appears heterogeneous (Ky), but the nuclear margin is distinct and intact (R). DMP 1317X

Figure 323. The same nucleus as in Figure 322 showing no demonstrable BP uptake by any karyoplasmic structure. The nuclear rim (R)

and the wall of the blebs (B) show BP accumulation. UVFM 1317X

Figure 324. The same nucleus as in Figure 322 and 323 showing a different focal plane. DMP 1317X

Figure 325. Two isolated liver nuclei incubated in the complete DNAase incubation medium for six hours at 37°C. DMP 1317X

Figure 326. The same two nuclei as in Figure 325 showing no demonstrable BP accumulation by any intranuclear structure. Bp uptake is discernible in the nuclear rims (R) and in the walls of the bleb (B). UVFM 1317X

Figure 327. An isolated nucleus sequentially treated with DNAase and 1M NaCl extraction. BP accumulation is apparent in the nuclear rim but not in intranuclear sites. The structure marked "R" is a crease in the nuclear rim. UVFM 1317X

Figure 328. The same nucleus as in Figure 327 showing nucleolar residue. DMP 1317X

Figure 329. The same nucleus as in Figures 327 and 328 showing a slightly lower focal plane. UVFM 1317X

Figure 330. An isolated liver nucleus digested with DNAase followed by exhaustive extraction with 1M NaCl. Nucleolar residue (nc) and a nuclear rim (R) are present. DMP 1317X

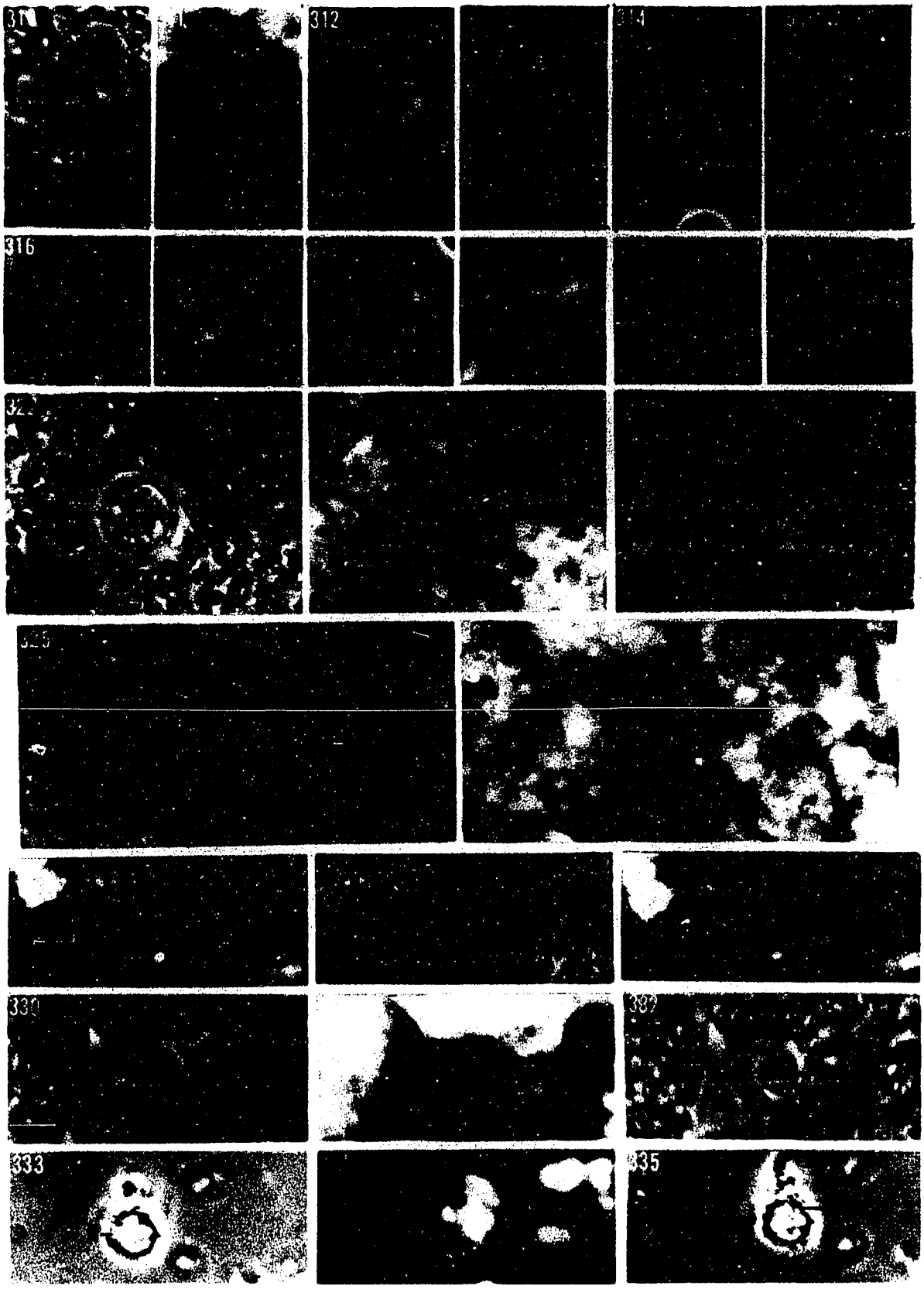
Figure 331. The same nucleus as in Figure 330 showing BP uptake by the nuclear rim (R) but not by any intranuclear structure. UVFM 1317X

Figure 332. The same nucleus as in Figure 330 and 331 showing a slightly different focal plane. DMP 1317X

Figure 333. An isolated liver nucleus sequentially treated with DNAase hydrolysis and with 1M NaCl extraction. A break in the nuclear rim (R) is seen. DMP 1317X

Figure 334. The same nucleus as in Figure 333 showing no apparent localization of BP within the confines of the nuclear rim (R). UVFM 1317X

Figure 335. The same nucleus as in Figures 333 and 334 showing a continuous nuclear rim (R) at the slightly different focal plane. DMP 1317X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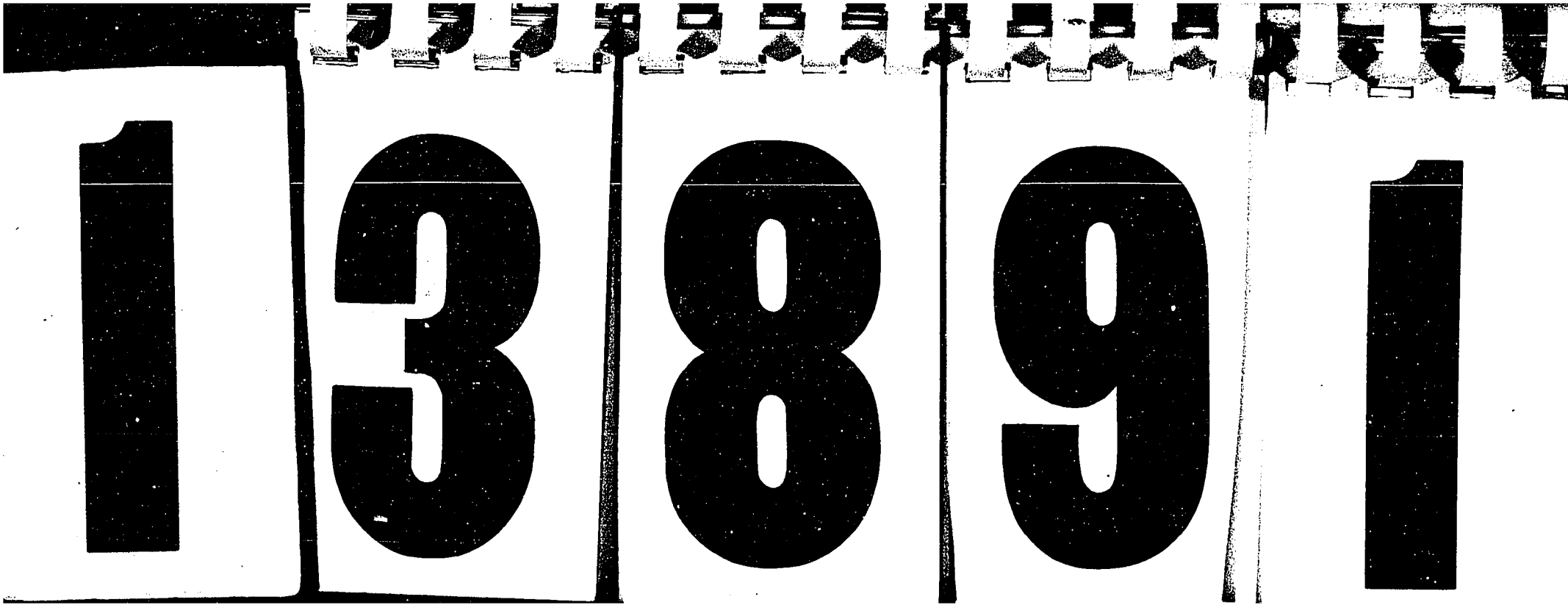
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