A HISTOLOGICAL APPROACH TO THE STUDY OF SUBLETHAL CYANIDE EFFECTS ON RAINBOW TROUT OVARIES

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A Thesis

in

The Department

of

Biological Sciences

Presented in Partial Fulfillment of the Requirement
for the degree of Master of Science at
Concordia University
Montréal, Québec, Canada

April, 1977

ABSTRACT

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In both an early and late summer experiment, young Salmo gairdneri were exposed for 20 days to assayed 0.01 and 0.02 mg/l HCN concentrations in continuously renewed water at 10.5°C, pH 7-8.

Histological examination of the ovaries at the light microscopic level revealed several oocyte abnormalities. These included delayed Balbiani dispersion and increased PAS-positive granules suggesting an interruption in utlization of protein and mucopolysaccharides within the cytoplasm and a subsequent effect upon yolk deposition. Oocytes displaying affected egg membranes demonstrated loss of cell contour and exaggerated ellipsoidal shapes. In both experiments, oocytes exposed mainly to 0.01 mg/l HCN displayed a unique form of atresia which was present in all stages of oocyte development. At the ultrastructural level, cyanide-exposed oocytes revealed within the mitochondria characteristics of both early and advanced pathological conditions at 0.01

and 0.02 mg/l HCN respectively. These observations suggested an interruption in the energy-producing mechanisms due to cyanide.

By the establishment of six arbitrary stages of oocyte development, the effects of cyanide on frequency distribution were quantitatively assessed. The early summer experiment revealed a significant 42% increase in stage 3 oocytes indicating fewer eggs developing beyond the Balbiani protein deposition. Ovaries exposed during late summer indicated 35-38% lower frequency of oocytes with secondary yolk (stage 5) as compared to controls along with significantly increased stage 3. This was especially evident at 0.01 mg/l HCN. Potential growth studies, based upon mean maximum diameters, indicated a significantly retarded growing rate in cyanide-exposed ovaries. The frequencies of major diameters revealed absence of the upper range limits after cyanide treatment.

These morphological and quantitative data reflect the sensitivity of rainbow trout ovaries to cyanide. In combination they suggest the possibility of a delay in spawning resulting in the release of eggs during unfavourable

environmental conditions or a decrease in the number of viable eggs available for reproduction.

Acknowledgments

I take this opportunity to express my appreciation to everyone who assisted me. I am grateful to Dr. Sylvia M. Ruby, who gave me the opportunity to accomplish this degree; aided by the National Research Council grant No. 240-232.

Sincere thanks to Dr. G. Leduc, Dr. P. Anderson, Dr. Wang and Dr. S. d'Apollonia. I thank my fellow graduate students and friends: D.G. Dixon, M. Speyer, D. Galley, S. Cheng and N. Hibberd for their assistance.

L'université de Québec à Montréal made available the use of the electron microscope.

A special thank you is extended to Mr. John Fotheringham of the Montreal General Hospital for his valuable technical advice. Dianne Cunningham kindly typed the manuscript. I am most indebted to my husband, Laurence Newton for his continual encouragement.

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Introduction

Cyanide has long been recognized as a potential threat to aquatic life. Undissociated and in compound form, it is frequently present in waterways as a result of various industrial processes. The electroplating, metal-finishing and mining industries are major contributors but effluent cyanides also occur from scrubbing gases at steel plants and from gas works and coke ovens (Germain and Vellog, 1975).

Recently, the occurrence of excessive cyanide wastes has been reported in Canadian waters. Bérubé and Gilbert (1971) recorded levels reaching as high as 10 to 30 mg/l HCN in mine tailing ponds and 0.01 mg/l HCN in northern rivers. This study has been stimulated over concern of these increasing levels. It is hoped that the histological findings of this study will contribute knowledge regarding the effects of cyanide upon the critical physiological functions of reproduction in female rainbow trout.

All efforts to control water pollution and ensure adequate water quality require some understanding of the parameters acting on the toxicant in the environment.

The lethal toxicity of molecular cyanide (HCN) varies with temperature (WPRL Report of the Director, 1967, p.62); (Brown, 1968, p.733), dissolved oxygen (Wuhrmann and Woker, 1955) and photo-decomposition (Burdick and Lipschuetz, 1950). PH affects the potency of the alkali metal salts of cyanide in solution since the extent of HCN formed is increased inversely with pH. In natural waters (pH 6.5 to 8.3) sodium or potassium cyanide hydrolyzes almost completely to HCN (Doudoroff, 1956).

Wuhrmann and Woker (1948); Doudoroff et al (1966) have demonstrated that the effective cyanide species to fish is the volatile hydrocyanic acid (HCN). The lethality of hydrocyanic acid is directed upon the respiratory chain, specifically at the cytochrome oxidase and succinic dehydrogenase sites (Lehninger, 1970, p.379). Inhibited electron transfer essentially renders cells incapable of utilizing blood oxygen (Jones, 1964, p.83).

Acute concentrations of simple cyanides to fish ranges as low as 0.02 to 0.06 mg/l CN for white crappie, Pomoxis annularis, and bluegill, Lepomis macrochirus, respectively (Renn, 1955) held in continuous flow systems.

Hardier species (Pimephales promelas) cannot withstand levels much beyond 0.24 mg/l CN (Doudoroff, 1956). Other workers (Herbert and Merkens, 1952) have shown that a concentration of 0.07 mg/l CN at 17.5 ± 0.5°C, pH 7.4 to 8.0, is fatal to rainbow trout, Salmo gairdneri. Their results indicated that survival time was closely related to the length of yearlings. Brown (1968) reported a 48-hour LC50 of 0.07 mg/l HCN for rainbow trout at 15°C, pH 7.7.

However, fish mortality may result indirectly from sublethal concentrations of cyanide. Several investigations reveal a general reduction in the ability to perform various physiological functions required for the successful survival of the species. Effects on swimming ability, osmoregulation growth, food consumption, embryological development and reproduction have been reported in the literature.

Neil (1957) found serious reduction in swimming ability of yearling speckled trout, Salvelinus fontinalis, at concentrations of 0.01 to 0.05 mg/l CN exposed 29 days. Total recovery was incomplete after 20 days. Similarly, Broderius (1970), showed a significant decrease in swimming time against a constant water velocity in coho salmon,

Oncorhynchus kisutch, exposed to 0.01 mg/l HCN.

Studies on osmoregulation in rainbow trout (Leduc and Chan, 1975) revealed that following a 28-day exposure period to various cyanide concentrations (0.01 to 0.037 mg/l HCN), treated fish introduced into 18.8 p.p.t. salt water showed higher plasma osmolarity and chloride concentrations compared to controls. Upon return to fresh water aquaria the poisoned fish exhibited a greater loss of chloride ions. Most of the effects occurred at 0.01 mg/l HCN with little intensification at higher levels.

In growth experiments by Speyer (1975), rainbow trout were placed in 0.02 mg/l HCN at 11 ± 0.5°C and an early depression of growth was evident, followed by an accelerated rate exceeding controls after 10 days. The work of Leduc (1966b) demonstrated that cyanide-treated cichlids fed unrestricted diets increased food consumption but converted food less efficiently than control fish. A pronounced effect was likewise determined for juvenile Salmo gairdneri with restricted food rations (Dixon, 1975).

Leduc (1976) showed impaired embryological development in Atlantic salmon, Salmo salar, exposed to levels of 0.01 to 0.10 mg/l HCN during the whole development. A high incidence of gross abnormalities was demonstrated in fry post cyanide concentrations as diluted as 0.01 mg/l HCN. Egg mortality surpassed controls 22 to 45% respective to the cyanide gradients. In another embryological study, Wilde and Crawford (1966) incubated Fundulus heteroclitus embryos in cyanide, (2×10^{-3}M NaCN). Embryos underwent normal cleavage and blastulation but post-blastula development was inhibited. Morphogenesis resumed normally after a one day lag upon removal from cyanide.

Relatively little attention has been focussed on chronic cyanide effects to fish reproduction. Pollutants such as cyanide may interfere with hormone production and control, translocation of energy stores or in some other manner impair normal gamete production. Such changes altering reproductive patterns of a population could lead to its decline or disappearance (Warren, 1971, p 122-125).

To date, only two studies of the effects of chronic cyanide poisoning on the reproduction of salmonoids could

be found in the literature: Broderius (1975) performed experiments using brook trout through reproduction and hatching. Combined values for both egg yield and per cent egg hatch were normalized to controls and the results indicated only 80%, 50% and 30% effective reproduction at 0.050, 0.100 and 0.300 mg/l HCN respectively. Histological observations of fingerling rainbow trout testes (Ruby and Dixon, 1974) showed that cyanide partially arrests mitotic division of spermatogonia at the metaphase stage. Exposure to 0.01 and 0.03 mg/l HCN in continuous-flow tanks for 18 days at 12.5°C revealed a 13% and 31.5% decrease in mitotic activity of developing germ cells compared to controls. Cellular damage was evident in spermatogonia accompanied by a high incidence of necrobiosis at the highest concentration.

Current literature indicates an increasing utilization of histological techniques to assess the impact of aquatic pollutants on fish reproductive organs. Atypical oocytes were observed in the ovaries of cutthroat trout, Salmo clarki, after exposure to the insecticide endrin (Eller, 1971). Although no abnormality occurred in testes, 31% of oocytes in early and late vesicular-yolk stages

differed from controls for fish at high concentrations. Oogenesis did not progress beyond late vesicular-yolk deposition. The effects of injected copper acetate and asphalt on ovarian activities in two fish species were investigated via histological examination by Khosa and Chandrasekhar (1972). Copper acetate was found to stimulate egg size while yolkier eggs with prominent granulosa were reported due to asphalt after a three month exposure interval. The work of Tafanelli and Summerfelt (1975) reported cadmium-induced changes in the gonads of goldfish, Carassius auratus. Production of ova was retarded or inhibited in fish receiving multiple cadmium injections.

These studies indicate that through histological techniques, the toxic nature of a compound may thus be proven and the extent of damage related to the different exposures and concentrations. Early indications of pollution may be detected prior to external manifestations.

Electron microscopy has been especially beneficial in elucidating subtle organelle responses to aquatic toxicants in several organs other than gonads. Hinton et al (1973a, 1973b) estimated the amount of kidney necrosis in channel catfish, exposed to methyl mercuric chloride and

localized the corresponding acid phosphatase reaction product in the liver. Electron microscopical observations on copper poisoning in winter flounder (Baker, 1969) confirmed vacuolation in gill epithelia with myelin-like figures, various unusual vesicles and identified chloride cells replacing mucous-producing cells.

The primary aim of this study was to determine any structural alterations in rainbow trout ovaries brought about by chronic cyanide exposure. A histological description of the gonad was prepared from light and electron microscopy. Light microscopical examination was based on classifications outlined after several authors (Beams and Kessel, 1973; Lewis and McMillan, 1965; Braekevelt and McMillan, 1967; Malservisi and Magnin, 1968). Both a qualitative and quantitative phase was necessary in prder to establish a baseline of normalcy and to appraise any damage incurred in the tissue subsequent to sublethal concentrations of cyanide as hydrocyanic acid. Oocyte ultrastructure and early vitellogenesis have been previously documented for several fish species (Yamamoto and Onozata, 1965) including rainbow trout (Beams and Kessel, 1973). Mitochondrial structure was examined in oocyte and follicular cells using

electron micrographs and compared to cyanide-treated fish.

Development of eggs in maturing ovaries generally enhances stress upon biochemical functions within rainbow trout, and this places a high energy demand upon the organism. Simultaneous exposure to sublethal hydrocyanic acid during this critical period imposes an additional stress upon developing ova, particularly during primary and secondary yolk deposition stages, and could detrimentally affect successful spawning of rainbow trout.

This project endeavors to establish by means of a histological approach, the normal ovarian developmental state of young rainbows, to describe and statistically correlate the differences due to cyanide at the light level and to evaluate any organelle changes that may occur at the ultrastructural level in response to chronic cyanide exposures.

MATERIAL, APPARATUS AND METHODS

Material

For this study, young rainbow trout, Salmo gairdneri (Richardson), were purchased from La Pisciculture du Lac a L'eau Claire Enr., St. Alexis-des-Monts, Maskinonge County, Quebec. One hundred and fifty fish varying in length from 12.7 to 15.2 cm were transported to the laboratory in plastic bags containing ice-water and pressurized with oxygen. Care was taken to assure uniformity of weight and size for the two experiments undertaken but due to summer growth the weights of the fish averaged 35.61 g (May) and 43.35 g (August) respectively for the first and second shipments.

The stock received at Concordia University (Sir George Williams Campus) was held in 80 liter tanks at a density of approximately 30 individuals per unit. Additional ice was necessary to slowly acclimatize the trout to the temperature of the flow-through system.

Trout chow (Purina, 40% protein) was immediately initiated and continued throughout the experimental period.

Apparatus

Water supply

City of Montreal water was piped into the laboratory and passed through an activated charcoal dechlorinator reducing the chlorine content to less than 0.01 mg/l. Chemical characteristics of the water used from May 1974 to September 1974 were provided by the City of Montreal Public Works Service, Waterworks Division and the critical data is tabled below:

Table 1. Chemical characteristics of City of Montreal water.

Monthly Mean	Alkalinity ($CaCO_3$) (mg/l)	Total Hardness (mg/l)	CO_2 (mg/l)	pH
May	81	117*	0.3	7.9
June	83	120	0.7	7.9
August	85	127	0.6	7.8
September	83	124	0.5	7.8
Year average	85	126	0.4	7.9

* Lowest concentration of total year

The inlet water was then supplied to the experimental apparatus by plastic PVC (poly vinyl chloride) piping into a main head tank. From here it was delivered to the holding facilities, via an aerated 10.16 cm i.d. Plexiglass column which provided rapid aeration and prevented the occurrence of air-supersaturation in cold water. By means of a graduated flowmeter (Manostat Corp., New York) the flow into individual tanks was maintained at a continuous rate of 1.1 l/min.

The temperature of the water reaching the testing tanks was regulated to 10.5 ± 1°C by means of an inline chiller (PC5HQ, Dunham-Bush of Canada Ltd.) and during the latter summer run, an auxillary portable unit (Blue-M Electric Co., Model Pcc-1355A-2) was submerged in the overhead reservoir.

Tanks

The fish were accommodated in translucent white, polyethylene tanks (Rosedale Plastics, Montreal) which measured 57.5 cm wide by 68 cm long by 42 cm deep filled to a volume capacity of 80 liters. These units were mounted on a common drainpan and each was fitted with a large-mouth standpipe and outlet tubing made of the non-reactive PVC

material. Covers were constructed with fiberglass mosquito screening framed in a Masonite pressed wood, resin-coated (Standard Boat Resin, Waldor Chemical Co., Pte. Claire) to limit leaching. An elbow on the left corner housed a stationary funnel draining into a weighted tubing on the floor of the tank.

From Mariotte bottles shelved above the tanks, the toxicant was introduced by 0.16 cm i.d. butyrate tubing which was supported alongside the water inlet hose (Leduc, 1966a). In this manner, total mixing of inflowing water and toxicant could be achieved without any interruption to the set-up during feeding, cleaning or sampling (See Figure 1.) after Dixon (1975).

The experimental assembly was curtained by black plastic sheeting to limit external disturbances. A 12-hour photoperiod was assimilated by a time-switch connected to a series of 40 watt fluorescent lights (Lifeline, Model F-40, warm white, Sylvania Can.).

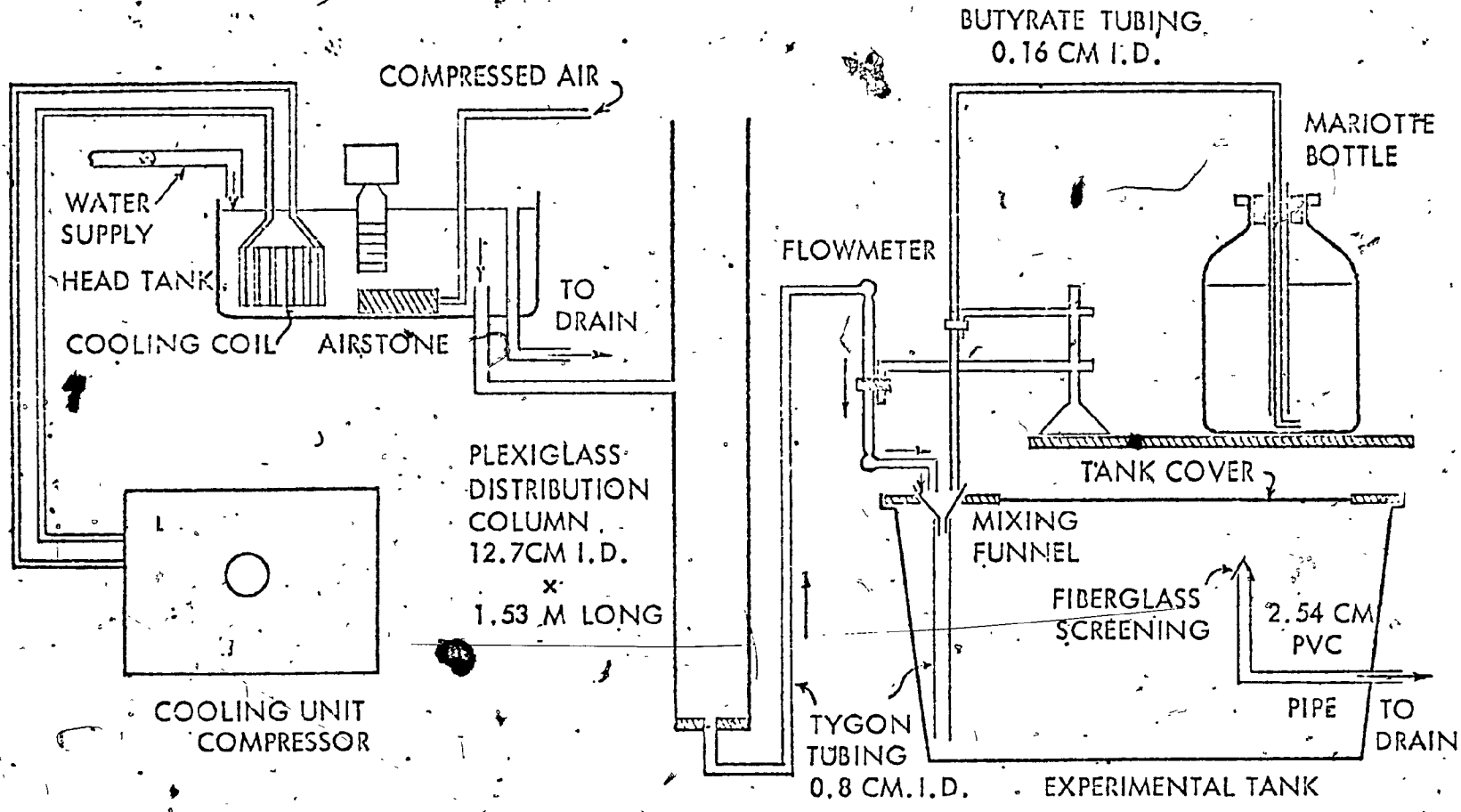


Figure 1. Schematic drawing of the experimental apparatus used for the continuous exposure of juvenile rainbow trout to sublethal concentrations of hydrogen cyanide, showing the heating and cooling systems, the direction of water flow, one experimental tank, and one Mariotte bottle for dispensing toxic solutions (D.G. Dixon, 1975).

Methods

In preparation for the cyanide tests, the fish were previously starved for 24 hours to assure the best results in weight grading. Fish of the desired range were randomly segregated under red fluorescent lighting to minimize stress produced by handling. Three tanks each containing 24 fish (males and females) were subjected to experimentation. Selected individuals were anaesthetized with MS222) tricaine methane sulphonate, Sandoz), dabbed dry to weigh to the nearest 0.01 g and branded with liquid nitrogen (Mighell, 1969) for future identification. They were returned to their original containers for completion of the acclimatization period before the commencement of cyanide exposure.

For the first experiment, the trout weights ranged from 34.79 to 36.43 g and the above described procedures were executed prior to the two week holding time. For the remaining experiment, weighing and branding were conducted one day before the addition of cyanide and these weights ranged from 42.76 to 43.90 g.

In the course of this adjustment period, identical conditions were maintained as those throughout the testing. The fish were fed a daily allotment of food in pellet form at a level of 2% total body weight. With sampling removal of 50% of the population this amount was simply reduced by one half.

Routine attendance to the apparatus involved daily cleaning of the tanks and repeated timings of flow rates. Residual chlorine was checked weekly by the orthotolidine method (Standard Methods. 1971) whereby the incoming water sample was matched in a colorimeter equipped with a fluorescent lamp assembly.

After the required acclimatization, the cyanide solutions were prepared according to Leduc (1966a). The dilution water flow rate of 1.1 l/min provided a 99% replacement of tank water every 5½ hours as calculated after Sprague (1973). On the evening before experimental day one, sodium cyanide was metered from Mariotte systems into the tanks at a rate of 2 ml/min to establish the concentration of 0.01 and 0.02 mg/l of cyanide as HCN (hydrocyanic acid).

The control group was similarily treated except for the absence of poison.

Every third morning the concentration of cyanide in the tanks was determined by the Epstein colorimetric method (Standard Methods, 1971) which is sensitive for microquantities of cyanide between pH 7 and 9 (Epstein, 1947). The stock standards cyanide was titrated at intervals with silver nitrate (1 ml = 1 mg CN, Harleco) but the reagents were prepared fresh with new assays. Tank cyanide concentrations were closely estimated from a standard curve obtained using a spectrophotometer (Spectronic 20, Bausch and Lomb) set at 620 mm wavelength. A reagent blank was used to zero the optical density following a 30 minute period of colour development. The test water was withdrawn by means of a volumetric pipette. The modification with butyl alcohol was found to be unnecessary in the relatively clear test waters.

Although it was not always possible to maintain the predicted concentration of cyanide in the tank, reduction was never reported more than 17% of the decided strength and when necessary the flow of cyanide stock solution was adjusted accordingly.

On alternate days, both the tank and reservoir waters were analyzed for dissolved oxygen. A sample taken at mid-depth was prepared as per Winkler's Method (Standard Methods, 1971) using the azide modification for interfering nitrites common in biological effluents. Sulphuric acid preserved the reaction and the precipitate settled in BOD bottles before the transmittance was recorded at 450 µm in the spectrophotometer. The concentration of dissolved oxygen was calculated from a standard curve outlined by Oulman and Baumann (1956) and modified by Dixon, (1975). Saturation never fell below 75% throughout either experiment.

The pH of the incoming water was regularly measured with a reference buffer standard (7.00 ± 0.01 at 25°C, Harleco) and calibrated on an electrode pH meter (Type PHM 28, Radiometer, Copenhagen). Readings fluctuated between 7.65 and 8.05 units with the test tanks slightly more acidic but remaining within this range. Sodium hydroxide was added to the Mariottes steadying the pH at 9 or more to limit cyanide stripping (Neil, 1957).

Experimental Design

Two similar experiments were performed during early and late summer, respectively, to evaluate the chronic

effects of cyanide on rainbow trout ovaries. On the 15th day of the test, gonads from 12 fish per tank were removed for histological examination and the remaining population was sampled at the end of 20 days. This histological project attempted to ascertain the seasonal state of control ovaries and to compare both quantitatively and qualitatively any significant changes in the cyanide-treated specimens. The data for 15 and 20 days were pooled.

Histology

Upon the termination of the designated experimental time, the fish were weighed and the dorsal nerve was severed. A mid-ventral incision was made from the gills to anal fin whereby the right gonad was quickly removed. Ovaries were fixed at 0°C in 1.25% gluteraldehyde buffered with 0.133M PO4 at pH 7.4 for 2 hours, cleared in buffer, and post-fixed for 2 hours in 2% osmium tetroxide and buffer (Galigher and Kozloff, 1971). Acetone dehydration was followed by embedding in Araldite resin (Ladd Ind.). One hour dessication eliminated air bubbles from the final infiltration of resin placed in Beem capsules and the tissues were oven-dried at 60°C for 36 hours prior to trimming. Half micron and silver sections were inverted on copper grids (Athene type, mesh 200).

Thick sections were stained in 1% toluidine blue saturated in sodium borate. The method was modified after Aczel (1975) to 3 minutes using a hot plate at 50°C. A staining scheme for EM grids was comprised of uranyl acetate (Reynolds, 1963) for 6 minutes and secondarily, lead citrate (Watson, 1958) for 15 minutes.

For light microscopy, the left gonad was simultaneously dissected into 2 pieces; one being placed in Helly's fluid (Culling, 1957) while the other portion was preserved in 4% formal-fish saline (Carleton, 1938; Hoar, 1966) for 24 hours.

Tissue was dehydrated in a gradient ethanol series from 70% to absolute for a total of 7 hours, cleared in toluene, infiltrated and embedded in paraffin (Tissue Prep, m.p. 56°C ± 0.5°C), Fisher Scientific Co.). Gonads prepared in Helly's solution were rinsed overnight in cold water and washed in ethanol-iodine to remove traces of mercury pigment prior to routine processing. All reproductive organs were aligned and cut longitudinally in cassettes.

Microscopic sections for the verification of female trout were cut at 5 microns and stained with 1% aqueous eosin and Harris hematoxylin.

Formalin fixation best maintained cell structure for measurements and counts. Blocks were sectioned into step-serial levels (Gold, 1966) in 10 equidistant intervals of 40 microns with each level having 5 8-micron sections. The total depth into the ovary was 800 microns. Feulgen reagent (de Tomasi modification) was employed in the periodic acid-Schiff (PAS) technique (Culling, 1957) counterstained in Harris hematoxylin for the localization of mucopolysaccharides and the slides were mounted with Permount resin.

Helly's-fixed portions displayed advantages for cytological features such as mitochondria identification (Altman Method in: Culling, 1957).

Thick (half micron) sections oriented the reference region for electron microscopy in addition to providing detail too thick to be otherwise discerned.

Histological Processing Equipment

The reproductive tissue was processed in a Tissuematon (Dual Unit, Fisher Scientific Co.) for the first experiment and a Technicon Ultra Processor (Technicon Int., Can.) for the second. Paraffin embedding was completed on a Tissue Tek II Embedding Center (Lab Tek, Model 4603, Fisher Scientific Co.). Slides for light microscopy were prepared using a rotary microtome (Spencer AO '820') while half micron sections were cut with glass knives on a Porter-Blum ultra microtome (Sorvall-MT-1).

Histological studies were done under a combination of Leitz binocular and orthoplan microscopes (Wetzlar, Ger.) fitted with micrometer discs (Whipple) for quantitative estimates. A camera (Leica, Wetzlar, Ger.) was attached to the column of the orthoplan for photographing histological sections. Thin sections were viewed and photographed at 6- KV with an electron microscope (Phillips 300).

For light micrography, Panatomic-X film (ASA 32, Kodak) was utilized and subsequently developed in Microdol-X (Kodak). Electron micrographs were photographed using fine grain film (FRP 426, Kodak) followed by developing in D-19

(1:1 dilution) for 5 minutes. Black and white photographs were developed in Dektol after the methods outlined by Dawes (1971) and Desilets (1974) and printed on Kodabromide and AGFA Brovira papers (grades 3,4,5).

Histological Analysis

The quantitative analysis adopted for this histological investigation was designed to obtain the maximum information on changes in egg distribution, frequency of stages and potential growth of developing eggs.

The growing eggs present during May through September were classified from all control fish. Six arbitrary stages of development were assigned based upon changes in profile size, cytoplasm, nucleus, membranes and follicular cells. A seventh class included several forms of degenerating (atretic) ova which occur naturally in the gonad.

Oocyte profiles were measured using an eyepiece micrometer. To obtain a survey independent of region, 10 of the best-fixed (Weibel and Elias, 1967, P. 141) control blocks were chosen with the section and level set by a table of random numbers. Due to the ellipsoidal nature, both the major and minor axes of the complete oocyte and its nucleus

were recorded.

Each diameter value was standardized by using the square root of the product of the greatest (major) diameter multiplied by the least (minor) diameter (Braekevelt and McMillan, 1967). The nucleus/cytoplasm ratios were computed and the number of nucleoli visible in an 8-micron section was counted. Total sample size was 120 measured control oocytes with calculated standard errors.

The corresponding histological features were described for each measured oocyte to mark the progress of egg maturation. The final class limits or stages were established by size in conjunction with cytoplasmic criteria. The stages of oocytes encountered on the surface edge or without the nucleus, were estimated by size and/or degree of yolk development.

A total of 74 cyanide-exposed oocytes were measured and described by the same procedures.

Systematic sampling throughout the ovary was initially necessary to determine the distribution of various egg stages. Step-serial levels recorded a (longitudinal)

depth of 800 microns into the ovary. This incorporated 75-100% of the left ovary segment.

Maximum growth, by size, within the ovaries was measured using the largest ova present at the various sampling times (Mathur and Ramsey, 1974). Every level and section was scanned and the major axis of the 10 largest oocytes per individual were measured and the means were computed.

Potential growth was determined as the difference in means of the maximum oocyte size from controls at Day 0, Day 15 and Day 20. No significant growth was detected over Day 15 to Day 20. Therefore, these two groups were consistently combined as a grand mean.

Cyanide-treated ovaries were similarly examined and measured for maximum and potential growth.

A pilot study tested the relationship between egg size and depth distribution within the ovary using the largest measured ova as markers. Cyanide-treated ovaries were likewise analysed for a homogeneous distribution.

The population of the seven stages (and thus all sizes) was tested for homogeneity. The frequency-distribution of the control stages was prepared by tallying oocytes falling randomly within the boundaries of an ocular grid. Any oocyte overlapping the outermost upper left and lower right corner square was not included in the counting (Weibel and Elias, 1967, P. 157). Initially, four areas for each 10 step-levels were recorded. All oocytes were viewed at 250 X magnification except oogonia which were checked under oil (1000 X).

Results of the previous growth and frequency findings in the controls permitted the application of random sampling techniques for further counts.

Since the frequency was also not apparently different among level locations, the counts were reduced to five random areas taken from the mid-region (5th level, 3rd section) of the ovary[1]. An activity index of egg production was devised by converting the individual stage means to percentage frequency, considering controls as 100% activity.

1. vernier co-ordinates were selected from a table of random numbers having out-focus to limit bias

The stage means and standard errors were calculated for controls and both cyanide concentrations. Significant differences among and between the three experimental tanks were tested by the student 't'-test using unequal sample n (Sokal and Rohlf, 1969, P. 220). A two-way analysis of variance and co-variance (SPSS-Manova) was applied to the data.

RESULTS

Oocyte Development

The ovaries of rainbow trout, Salmo gairdneri, are paired, elongate organs situated in the posterior body cavity; dorsal to the gut and ventral to the swimbladder. Ripe eggs are shed into the body cavity and pass to the exterior by folds forming a duct at the genital pore (Anderson and Mitchum, 1974, P. 78).

Active ovaries possess a reserve stock of germ cells ready to complete a new sexual cycle, even immediately after a spawn. The ovary is covered by a thin layer of epithelium, the mesovarium (Patt and Patt, 1969). Lying beneath there is a thin mesenchymal connective tissue named the tunica albuginea (Figure 2.). This vascular layer supports the gametes confined to the lamellar folds which project ventrally and laterally into the ovarian lumen (Figure 3.). A division into cortex and medulla is lacking. Mitotic division of the oogonia on lamellae generates the various stages of maturing oocytes. A homogeneous population of all stages is found to be distributed throughout the ovary, except at the extreme anterior, posterior and peripheral regions which demonstrate a high incidence of atretic

follicles and young oocytes (Figure 4.).

The younger developmental stages present over a period extending from spring (May) to fall (September) were defined by six classes. In general, growth begins with a vast increase in proteinaceous cytoplasm closely followed by yolk formation.

Stage 0

The resting oogonium is near-spherical and small (diameter 4-6.5 microns) with little specialization (Figure 5.). A thin rim of pale basophilic cytoplasm surrounds the dark-staining nucleus which occupies about 76% of the cell (See Table 2.). No nucleoli are visible but mitotic figures may still be encountered in recently divided oogonia (Figure 6.). Cell membranes, nuclear membranes and follicular cells are absent. Oogonia usually occur in "nest" associations embedded in stromal connective tissue as shown in Figure 6.

Stage 1

At this stage, the diameter of the cell varies from 6.5 to 20 microns but the primary oocyte remains within the nest (Table 3.). The opaque cytoplasm is lightly basophilic and without distinct margins (Figure 7.).

Table 2. Dimensions of control germ cells and nuclei during representative developmental stages in Salmo gairdneri females.

Stage	CELL Diameter Mean ±	CELL Range	NUCLEUS Diameter Mean	NUCLEUS Range	Nucleus/ Cytoplasm
	μm	μm	μm	μm	%
0	4.65 ± 0.13	3.74 - 6.25 (S) 4.00 - 6.33 (L)	3.57	2.80 - 4.17 (S) 2.92 - 5.81 (L)	76.25
1	8.91 ± 0.46	4.15 - 10.00 (S) 6.64 - 15.00 (L)	4.66	2.50 - 4.98 (S) 3.75 - 6.64 (L)	57.87
2	55.83 ± 2.99	21.82 - 76.36 (S) 36.00 - 79.08 (L)	33.64	20.91 - 45.45 (S) 25.60 - 52.72 (L)	55.77
3	102.41 ± 4.35	70 - 136 (S) 80 - 144 (L)	57.08	36 - 76 (S) 39 - 96 (L)	56.38
4	166.85 ± 5.77	88 - 188 (S) 160 - 228 (L)	90.69	61 - 112 (S) 82 - 148 (L)	50.05
5	247.16 ± 5.61	176 - 280 (S) 252 - 340 (L)	96.23	44 - 116 (S) 56 - 176 (L)	38.73

Diameters are standardized as the square root of the short axis (S) multiplied by the long axis (L). Standard errors were calculated for cell diameter. Sample size was 20 per stage.

@ microns- (μm)

Stage	Class Range (Am)	Nucleus	Nucleoli Mean Number/8-Am Section	Cytoplasm	PAS	Membranes	Follicles
0	4 - 6.5	Round, strongly basophilic	None visible	Rim, pale basophilic	-	Not clear	None apparent
1	6.5-20	Round, large, pale	None distinct from chromatin	Opaque, lightly basophilic	-	Distinct nuclear, indistinct cytoplasmic	Investing cells in stroma
2	20 - 80	Ellipsoidal, eccentric	9 large, few scattered	Strongly basophilic Balbiani idiosome and pallial layers	-	Nuclear and cytoplasmic, developing zona	Incomplete squamous follicle layer
3	80 -160	Ellipsoidal, similar	21 mixed sizes, migrate to periphery	Dispersing and fragmentation of Balbiani body, primary yolk vesicles	+	Completed zona pellucida	Thin, complete simple follicle layer
4	160 -240	Ellipsoidal, central, indented	35 small, peripheral	Secondary yolk vacuoles in nuclear and peripheral bands	++	Thickened zona pellucida, nuclear indented	Inner layer epithelial cells, outer thecal cells, both single squamous
5	>240	Irregular, moves to one pole	Up to 53, small, follow nuclear outline	Coalesced yolk globules, acidophilic	++	Wavy, thick zona pellucida, broken nuclear	Follicular layer cuboidal

Note: (-) negative and (+) positive reaction to periodic acid-Schiff stain.
Total sample size was 20 eggs per stage.

microns- (mm)

Table 3. Summary of morphological criteria defining arbitrary stages of egg development for two experiments extending from May to September.

The nucleus is larger than in oogonia, has a definite nuclear membrane and nucleoli are not distinguishable from the diffuse chromatin. The primary oocyte displays the extended "lampbrush" chromosomes which have entered the leptotene (prophase) phase of meiosis (Schreck, 1974, P. 15).

An incomplete squamous layer of investing cells occupies the spaces among the nested oocytes (Figure 7.). It is composed of spindle-shaped cells which gradually enclose the oocyte and later differentiate into the follicular layer.

Stage 2

The stage 2 oocyte increases in size up to 80 microns in diameter and both cell and nuclear contours are ellipsoidal (Figure 8.). This shape persists throughout the following stages.

A juxtanuclear Balbiani body [2] first appears in the cytoplasm. It has a duplex arrangement consisting of a non-basophilic core, the idosome, which is encircled by a deeply basophilic pallial layer (Beams and Kessel, 1973) illustrated in Figure 8.

2. alternative terminology in Appendix A

The patterned pallial substance contains a fibrous, network material and forms concentric rings outside the nucleus (Figure 9.).

An eccentric nucleus displays faint chromatin which loses affinity for stains. The nucleus is bordered by a distinct membrane (Figure 8.). One prominent nucleus is frequently located centrally while several smaller ones are scattered over the nuclear matrix (Figures 8,9.).

During this stage, thin egg membranes gradually encase the oocyte. The investing cells form an incomplete layer of delicate, flattened epithelial follicular cells (Figure 9.).

Stage 3

The oocytes of this stage have a diameter range between 80 and 160 microns. With cytoplasmic growth, the pallial substance too increases in mass and disbands towards the ooplasmic periphery (Figure 10.). The idiosome material migrates between the nucleus and cell border. Gradually the Balbiani body fragments (Figure 11.) and totally disappears by the end of this stage. Remaining peripheral ooplasm is frothy in appearance (Figure 12.) due to vacant

droplets of primary yolk vesicles (small vacuoles) washed out in ethanol preparation. These increase in number and later fill the entire oocyte. Since a high variation existed among the females, a moderate amount of forerunner secondary yolk may be present at this stage. Similarly, primary yolk may appear as early as stage 2.

The completion of three egg ensheathments is synchronized with yolk development. Primarily, there is an indistinct plasma egg (vitelline) membrane closely adherent to the egg surface. Outside lies the somewhat thicker zona pellucida (Anderson and Mitchum, 1974, P. 79). This refractile band (Figure 12.) is hyaline-like, non-cellular and strongly PAS-positive. Outermost, the investing cells (fibroblasts and reticular cells) form a single layer of squamous follicle cells which is more clearly observed at a later stage. The follicle cell nucleus is sausage-shaped and uniformly basophilic (Figure 12.).

During stage 3, the nucleus/cytoplasm ratio is reduced to 56% (Table 2.). The nucleus resumes the lampbrush type chromosomes (Figures 12,13.). A multiplication of the nucleoli has occurred averaging 21 in number per 8-micron section and they migrate to the perimeter of the nuclear

membrane (Table 3.). Clusters of PAS-positive granules congregate directly outside the nuclear membrane as indicated in Figure 13.

Stage 4

The diameter by this stage increases to 240 microns in profile. Small vesicles of primary yolk continue to spread across the entire ooplasm. Strands of PAS-positive granules radiate from the nuclear region and indent the nuclear membrane (Figure 14.). In Figure 14, beads of mucopolysaccharide-bound vacuoles form a distinct band on the ooplasmic periphery which fuse with the primary yolk vesicles resulting in deposition of secondary yolk (Braekevelt and McMillan, 1967). The zones of combined vacuoles are also evident outside the nucleus and press inwards on the fragile nuclear membrane (Figures 12,14,15, 16.). The cytoplasm becomes highly acidophilic and reacts positively to periodic acid-Schiff due to the production or accumulation of secondary yolk.

Only 50% of the cell is occupied by the central nucleus - again revealing extended and obvious chromosomes (Figure 16.). An average of 35 nucleoli of mixed stages

are positioned at the nuclear boundary (Table 3.).

The follicle cells consist of two single layers: an inner layer of follicular epithelial cells and an outer layer of thecal cells (granulosa). The zona pellucida appears between these two simple layers as demonstrated in Figure 17, and confirmed later by electron micrographs.

Stage 5

The most mature oocytes present in the sampled ovaries grow beyond 240 microns to a maximum 340 microns in diameter (Table 3.). Large secondary yolk vacuoles fill the entire cytoplasm (Figure 18.) and push the remaining traces of primary vesicles towards the periphery. The yolk vacuoles coalesce into irregular globules (Rajalakshmi, 1966) but retain the banding effect both at the cell periphery of the cell and haloing the nucleus (Figure 18.). At the end of this period, these globules occupy the entire cytoplasm.

The pressing yolk results in an involution and gradual loss of nuclear membrane. The nucleoli loose their peripheral position and disperse along the broken nuclear envelope(Figure 17.). Up to 53 nucleoli are present but are

typically small and stain lightly. Lampbrush chromosomes persist in the nucleus as it begins to shift to one pole. The nucleus/cytoplasm ratio is further decreased to 38% (Table 2).

Thecal and epithelial follicular cells enlarge to a cuboidal shape, each remaining as a single layer (Figure 18.). The wavy zona pellucida thickens slightly and intensifies in colour reaction to PAS.

Atresia

Some immature oocytes undergo a natural degeneration procedure which may occur at any stage in their differentiation. Atresia was present in all (control and cyanide-treated) ovaries viewed. It was most common in stages 3,4, and 5 of oocyte development.

The most widespread form of atresia, pre-ovulatory atresia (Zuckerman, 1962, P.248), involved the elimination of an egg and its associated membranes. The earliest signs are demonstrated in Figure 19. where a simple ridge of peculiar yolk vacuoles occur at the cell periphery.

Hypertrophied follicle cells multiply and act as phagocytes (Braekevelt and McMillan, 1967). The follicular phagocytes contain conspicuously large, indented nuclei which are pale-staining (Figure 20.). They congregate outside the zona pellucida which eventually loses its smooth contour, ruptures and permits entrance of the phagocytes to the interior (Figure 20.). Occassionally, the blunt cytoplasmic processes of phagocytes reveal undigested granules from the engulfed yolk. An empty ball of collapsed cells results from persistant involution at the oocyte periphery as the ground substance is simultaneously and completely resorbed (Figure 21.). Late atretic sites display rich vascularization and the scarred region readily becomes reorganized into general stroma.

Non-hypertrophic atresia was rarely identified in control, pre-ovulatory ova. It does not involve the swelling of follicular layer and membranes. Yolk reverts back to a finely granular texture with a basophilic affinity. Cyanide-influenced ovaries displayed a similar yet unique degeneration to be compared in detail under the pathology section to follow.

Post-ovulatory atresia was another form of atresia present in rainbow trout ovaries. Only a few ovaries were sufficiently mature to demonstrate the hollow shells left behind by shed ova (Figure 22.). The remaining stroma is extremely loose and convoluted. The normal-looking follicle cells are removed and replaced by rows of younger developing oocytes.

Pathology

Ovaries were examined after the exposure time in cyanide, and changes in oocyte structure were detected by light and electron microscopes. Examination of paraffin sections exhibited a number of abnormalities.

In both experiments, a unique morphological aberration was apparent whereby deep depressions in the ooplasm consequently resulted in oocyte lysis. Ovaries previously exposed to 0.01 mg/l HCN showed the highest incidence of damage, especially in Experiment 1.

The process was initiated with the retreat of acidophilic cytoplasm and loss of granulation and yolk vesicles when present. The resulting ooplasm appeared dull and opaque and a variable number of dark-staining

nucleoli became detached from the shrinking nuclear membrane (Figure 23.). Enlarged, amoeboid macrophages infiltrate the egg surface and interior. Their typically small, dark nucleus is most apparent when the macrophages entrench themselves in a row of vacuoles (Figures 24,25.). As yolk ingestion proceeds, the eroding action produces depressed furrows in the ooplasm (Figure 25.) until concentric swirls of clear furrows eventually replace the entire ooplasmic mass (Figures 26,27.). Separation of egg membranes and the associated follicular layer (Figure 27.) may alter the oocyte contour. Occasionally a few phagocytes occur on the egg boundary (Figure 27.).

These aberrations were recognized in most stages of oocyte development as indicated in Figure 28.). The degree of damage was not confined to any particular oocyte size or stage. Two ovaries exposed to 0.02 mg/l HCN demonstrated the earliest signs of breakdown as described for the lower cyanide concentration.

The above process of oocyte degeneration (and elimination) is a form of atresia unique within cyanide-contaminated ovaries. Pre-ovulatory atresia previously

described for controls is present as well in cyanide-treated ovaries, but differs considerably. Phagocytic follicle cells with large, pale and indented nuclei denote pre-ovulatory atresia (Figure 29.). These cells retain their peripheral position as the cytoplasm is digested inwards. The zona pellucida ruptures and this facilitates phagocyte invasion to the interior (Figure 30.) until a complete collapse of the oocyte ensues. In contrast, furrow formation as described above for cyanide treated ovaries, begins with an infiltration of macrophages spanning a large area within the ooplasm (Figure 31.). The characteristic macrophages contain a small, dark nucleus (Figure 32.). Total collapse of an oocyte proceeds with the lifted membranes generally remaining intact (Figures 31,32.). Peripheral phagocytes (Figure 32.) continue resorption of the scarred tissue.

Other effects of sublethal, chronic cyanide include alterations in the shape of many oocytes. Figures 33,34. illustrate an exaggerated ellipsoidal contour that is prominent in ovaries exposed to both test concentrations of cyanide. Experiment 2. revealed a greater occurrence of these ellipsoids. Although a low incidence occurred in

control fish, only the earliest deformation was apparent and it was restricted to the peripheries of the ovary. The extent of damage was not intensified with increased cyanide concentration, but the ellipsoid population extended from the peripheral extremities to the interior regions of the ovary.

Seriously deformed oocytes were also noted in a few cases of cyanide-exposed ovaries. Irregular bulges of cytoplasm and displaced nuclear materials were encountered as pictured in Figure 35.

Frequently the shape-affected oocytes showed almost complete separation of membranes from the egg surface (Figures 33,35.).

The loss of elasticity was demonstrated in many other cyanide-treated oocytes by extensive thickening of the adhering egg membranes. The hypertrophied and wavy zona pellucida (Figure 36.) renders hazy, improper resolution of these oocytes.

The majority of the two types of affected oocytes were classed into stage 4 or 5 by size consideration alone.

Their cytoplasmic development was not equivalent to control oocytes of similar dimensions.

Throughout Experiment 1, only the initial steps of yolk deposition were prevalent (Figure 37.). The primary effect of cyanide in oocytes of comparable size, appears as a lag in the disappearance of the Balbiani body. Figure 38. illustrates the patchwork effect of basophilic fragments migrating from the nucleus.

During Experiment 2, late stage oocytes displayed a peak period of secondary yolk development (Figure 39.). In the cyanide-contaminated oocytes, large accumulations of granules stagnate outside the nucleus (Figure 40.) and stain brilliantly with periodic acid-Schiff. Dispersion of these granules into the peripheral cytoplasm is abnormal and may be blocked as early as stage 3 (Figure 40.). Malformation of secondary yolk vacuoles reduces the mobilization to the cell border (Figure 41.). The normal banding of coalesced yolk globules is inhibited and results in an irregular distribution (Figure 41.).

Ultrastructural Observations

Several control and cyanide-treated ovaries were examined under the electron microscope. In late-stage oocytes, the follicular cells were confirmed as an inner epithelial layer and an outer thecal layer. Both layers appeared non-stratified. The thick, acellular zona pellucida was identified between the follicular layers (Figure 42.).

The ooplasm within control oocytes demonstrated a wide variety of bizarre forms of mitochondria. They varied from round to long and coiled (Figure 43.). The active elongate mitochondria were commonly characterized by the typical double membrane but revealed an irregular contour (Figure 44.). The inner membrane displayed transverse, tubular cristae of variable lengths extending into the homogeneous matrix. Dense granulations were apparent in the finely granular matrix (Figure 44.). (See chart 1.)

Control follicular (epithelial) cells consistently contained round or oval mitochondria which were bounded by a smooth-contoured, outer membrane (Figure 45.). Parallel tubular cristae were apparent within the colloidal ground substance. Small, dense matrix granules were generally

Normal (0.00 mg/1 HCN)	Early Injury (0.01 mg/1 HCN)	Advanced Injury (0.02 mg/1 HCN)
various shapes (round, oval, coiled).	slight swelling	severe, cloudy swelling
double membrane, smooth or irregular	hypertrophy of membranes interspace	ruptured, discontinuous membranes
parallel, tubular cristae	reduced, vesicular cristae	loss of cristae
small, dense matrix granules	loss of matrix granules	appearance of dense matrix particles

Chart 1. Summary of the observed characteristics of normal mitochondria in oocytes and follicular cells. Early and late signs of injury observed after cyanide treatment are also listed.

observed.

Ultrastructural examination of cyanide-treated oocytes revealed subtle effects on the mitochondria of both the ooplasm and follicular cells. Early structural alterations after 20 days exposure at 0.01 mg/l HCN in the follicular mitochondria, included increased matrix granules, laminations and ruptures of the double membrane frequently resulting in a release of the internal contents into the surrounding cytoplasm (Figure 46.).

Follicular cell oocytes at 0.02 mg/l HCN exhibited swelling of the entire mitochondrion, and hypertrophy of the interspace between the membranes (Figure 47.). Breakdown and vesiculation of the cristae accompanied by a loss of matrix granules were observed after the 20-day treatment.

After 0.02 mg/l HCN exposure, affected mitochondria within the central ooplasm appeared severely hypertrophied with discontinuous membranes (Figure 48.). The cloudy matrix contained aggregations of dense particles and a reduction in the number of cristae was obvious (Figure 48.).

Feeding and Wet Weight Observations

In both experiments, the fish quickly acclimatized to the tank environment and resumed feeding within two days upon arrival at the laboratory. Introduction of the toxicant resulted in stimulated swimming activities. After the fourth or fifth day, the fish held at 0.02 mg/l HCN exhibited lethargic, surface swimming accompanied by a decline in food intake, most pronounced in the first experiment. A similar response was evident after 8 to 10 days in fish exposed to 0.01 mg/l HCN.

A minimal number of fish mortalities occurred during both experiments (Table 4.). Control mortality was attributed to fungal infection and specimens were removed upon loss of equilibrium. Disease was almost eliminated in the treatment tanks after several days of cyanide exposure.

At the 15-day sampling, the number of females was not large enough (less than 5 individuals) to provide a complete set of data for statistical analysis. Again the 15 and 20-day mean values were combined for t-test analysis.

Table 4. shows the wet weight distribution between the spring and late summer experiments. It also compares

Expt. No.	Cyanide Conc. (mg/l HCN)	Sample Size (n)	No. Mortality in Expt.	Initial Mean Wet Weight (g)	S.E.	Final Mean Wet Weight (g)	S.E.	't' value Weight Change	't' value Final Control Toxicant
1	0.00	25	0	36.43	1.08	38.13	1.37	0.97	
	0.01	25	1	35.60	0.99	36.17	1.35	0.35	1.03
	0.02	25	3	34.79	0.47	31.46	0.61	4.50	4.230
2	0.00	24	1	42.76	1.29	51.78	2.92	2.67 *	
	0.01	24	0	43.90	1.05	51.00	2.939	2.94 *	0.22
	0.02	24	1	43.40	0.95	50.82	2.18	3.170 *	0.26

* $2.021 > p > 2.000$
Weight changes are combined 15 and 20 day means.

Table 4. Wet weights and incidence of mortality among control and cyanide-treated rainbow trout (males and females) during Experiment 1. (May) and Experiment 2. (August).

the initial to final wet weights for each experiment. The control initial wet weights were significantly different from Experiment 1. and 2. means of 36.43 ± 1.08 g and 42.76 ± 1.29 g respectively. Summer hatchery growth accounted for the weight increase. After 20 days exposure in Experiment 1., no significant change occurred in the final wet weights for either control or 0.01 mg/l HCN tanks. Final mean wet weights of fish exposed to 0.02 mg/l HCN were significantly different from controls and from initial mean recorded for this tank (See Table 4.). This weight loss was consistent with the noted change in feeding habits. In Experiment 2., all tanks exhibited significant gains in mean wet weights over the 20 days duration. No significant difference was evident in final means between controls and cyanide groups despite a slight food rejection observed at the highest concentration.

To test for any different effects of HCN on the growth of females, the mean wet weights of females were separately analysed in Table 5. These results show that whereas the growth of the pooled samples of males and females (See Table 4.) showed a significant effect of cyanide only at 0.02 mg/l HCN in Experiment 1., the control females grew faster and were much more affected by cyanide. Their wet weight gains were lower at 0.02 mg/l HCN in Experiment 1. and at 0.01 and 0.02 mg/l HCN in Experiment 2.

Expt. No.	Cyanide Conc. (mg/l HCN)	Sample Size (n)	Initial Mean Wet Weight (g)	S.E.	Final Mean Wet Weight (g)	S.E.	't' value Weight Change	't' value Toxicant Control
1	0.00	9	36.87	1.05	39.72	1.93	1.31	1.86
	0.01	12	33.63	0.71	34.83	1.59	0.51	
	0.02	12	34.89	0.59	30.87	0.72	3.19***	4.49 ***
2	0.00	8	47.45	1.75	63.74	3.59	4.08***	2.32*
	0.01	13	44.58	1.41	51.88	3.34	2.05	
	0.02	8	44.51	1.61	52.31	4.05	0.97	2.11*

*0.05 > p > 0.02; **0.02 > p > 0.01; ***0.01 > p > 0.001

Note: $\frac{E_1 \text{ Control (initial)}}{E_2 \text{ Control (initial)}} = 6.08$ ***

Table 5. Wet weights of separated female control and cyanide-treated rainbow trout.

In Experiment 1., the control females did not show significant growth over the 20-day exposure period, but the wet weight increase in Experiment 2. females was highly significant.

Effect on Frequency of Stages

A wide variation in the rate of ovarian maturation was observed within fish of an initially close weight and length range. Several complements of ova develop concomittantly within the ovary. One or two stages are predominant, however, at any one sampling period and the specific stage changes with seasonal growth. Figure 49. demonstrates the relative proportion of stages in the control fish and the incidence of all types of atretic follicles. A comparision of the two experiments reveals that the majority of oocytes present during the spring (Experiment 1.) were at stage 3 , whereas the late summer (Experiment 2.) stage peaks shifted to stage 4. The second experiment exhibited a higher frequency of stage 5 oocytes and overall depressed oogonia production (Figure 49.).

Mean frequency values for both control and treated fish are presented in Table 6. All stages were significantly different ($0.05 > p > 0.02$) between the two experimental control groups.

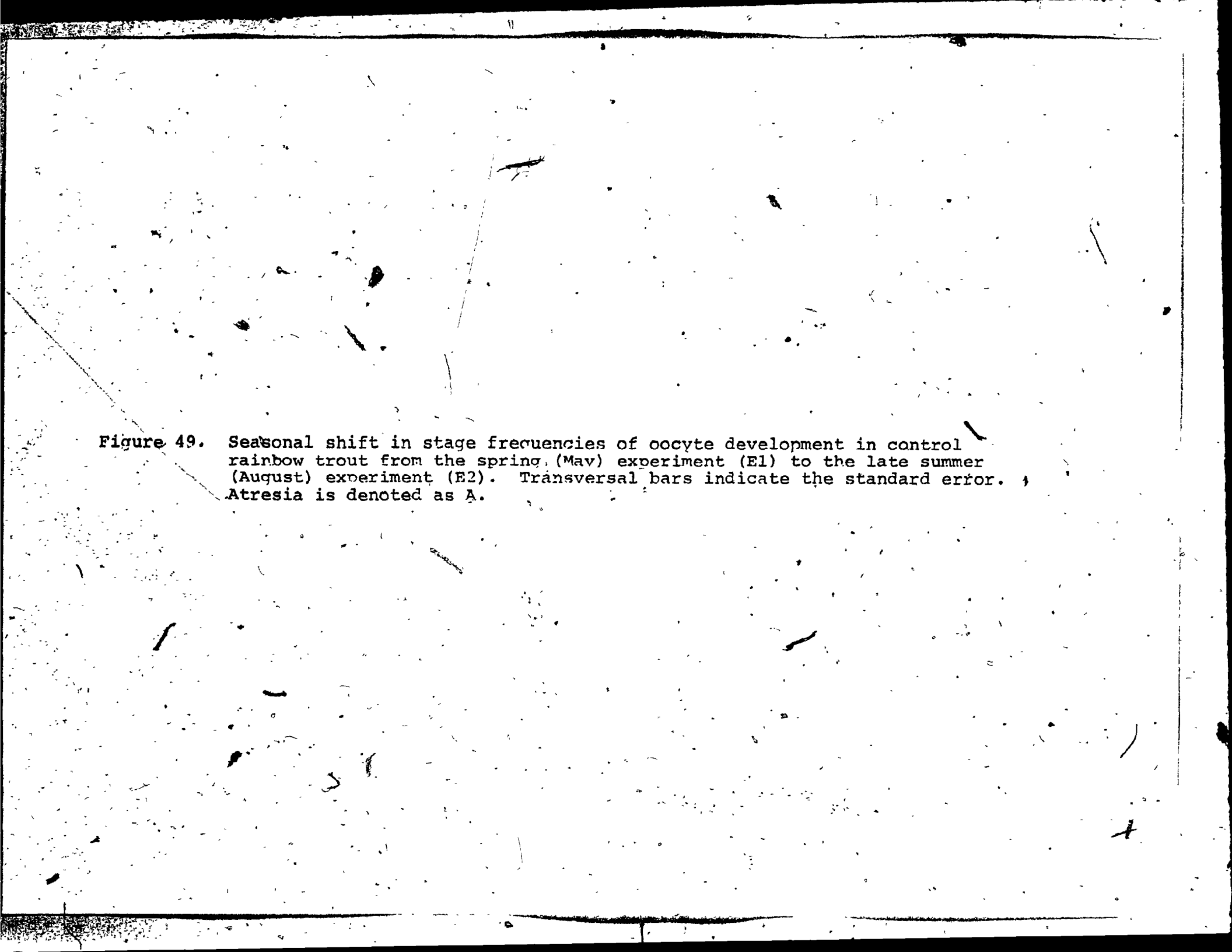
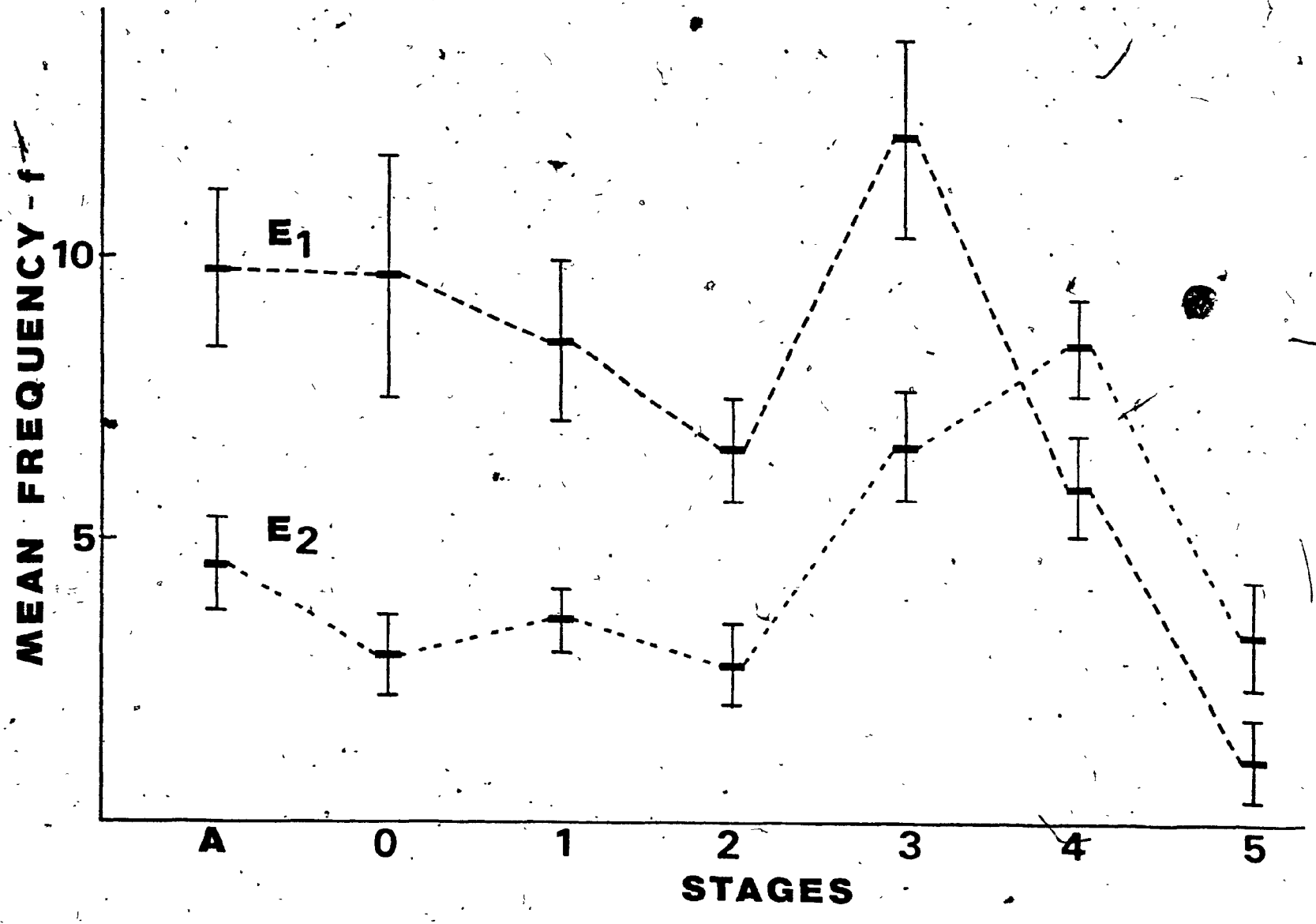


Figure 49. Seasonal shift in stage frequencies of oocyte development in control rainbow trout from the spring (May) experiment (E1) to the late summer (August) experiment (E2). Transversal bars indicate the standard error. Atresia is denoted as A.


MEAN FREQUENCY - f
10
5
E1
E2
A
0
1
2
3
4
5
STAGES

Expt. No.	Stage	Tank 0.00 mg/l HCN	S.E.	Tank 0.01 mg/l HCN	S.E.	Tank 0.02 mg/l HCN	S E.	't' value 0.01/0.00 mg/l HCN	't' value 0.02/0.00 mg/l HCN
1	0	9.56	2 11	10.08	1.72	13.92	2.20	0.20	1.42
	1	8.44	1.38	7.83	1.74	10.50	1.97	0.24	0.81
	2	6.56	0.92	8.33	1 84	9.50	1.75	0 78	1.36
	3	12.00	1.74	14.53	1.27	17.00	1 50	1.27	2.24 **
	4	5.89	0.89	6.00	0 88	5 67	1.10	0.09	0.15
	5	1.11	0.69	0.17	0 82	0.08	1.49	1.09	1.81 *
	A	9.67	1.39	8.58	0.56	7.42	0.79	0 74	1.55
2	0	2.88	0.68	7.31	1.59	3 38	0.62	2 11 **	0.56
	1	3.50	0.53	5.54	1.14	6.00	2.83	1.35	2.24 **
	2	2.75	0.69	4.23	0.66	1.75	0.94	1.50	0.88
	3	6.63	0.95	10.62	1.11	8.13	0.93	2.53 **	1.17
	4	8.34	0 83	8.85	0.54	9.75	1.40	0.56	0.88
	5	3.25	0.96	1.15	0.59	1.25	0.56	2.05 *	1.89 *
	A	4.50	0.83	9.08	0.80	8.50	1.09	2.15 **	2.99 **

* $0.05 > p > 0.02$; ** $0.02 > p > 0.01$

S E. standard error A-atresia

Table 6. Mean frequencies of oocyte stages based on five random areas per fish. Significant differences among means of controls and cyanide tanks are indicated for both experiments.

Stages 3 and 5 were significantly different at 0.02 mg/l HCN in Experiment 1. when compared to control means. In Experiment 2., 0.01 mg/l HCN was highly effective in stage 0,3,5 and atretic follicles. The 0.02 mg/l HCN concentration exhibited significant differences from controls in stages 1,5 and atresia (Table 6).

In the frequency histograms of Figures 50a and 50b, the control means per stage are expressed as 100% . The reproductive activity in the cyanide-exposed ovaries is indexed in Table 7. as percentage frequency of control stage activity (100%).

Figures 50a and 50b indicate the reproductive activity shift in the proportion of the different stages after a 20-day exposure to hydrocyanic acid. No significant change in stage frequencies resulted from 0.01 mg/l HCN exposure in Experiment 1. At 0.02 mg/l HCN, stage 3 activity is 41.67% higher than controls. A significant decrease occurs in stage 5 at the same concentration (Figure 50a). At the highest concentration in Experiment 2., the peak values (Figure 50b) remain at stages 3 and 4 as in controls but stage 1 (primary oocyte) production is significantly increased by 71.43% as well as

Figure 50a. Histograms showing the activity of oocyte developmental stages and (A) atretic follicles in control and cyanide-treated fish during Experiment 1.

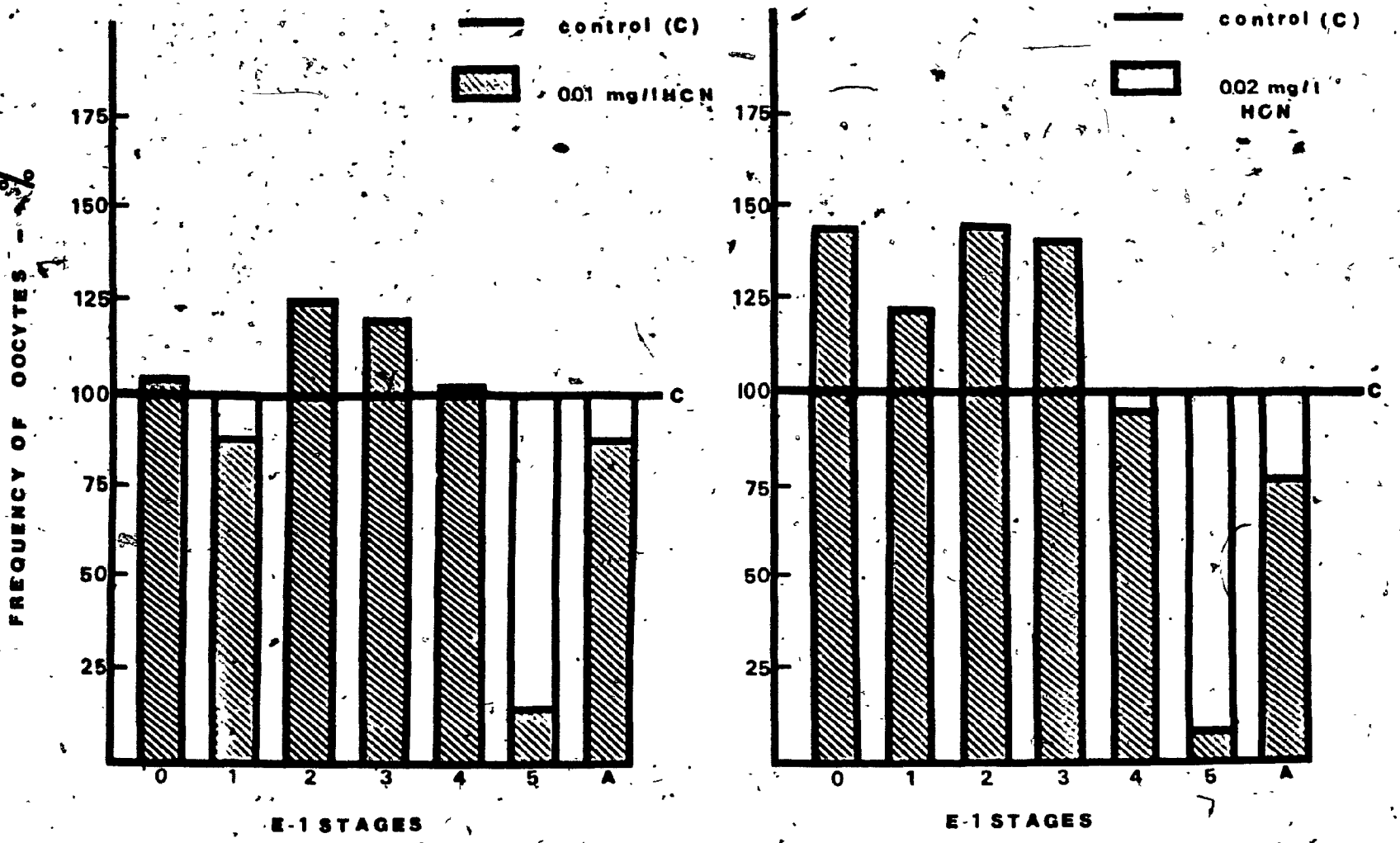
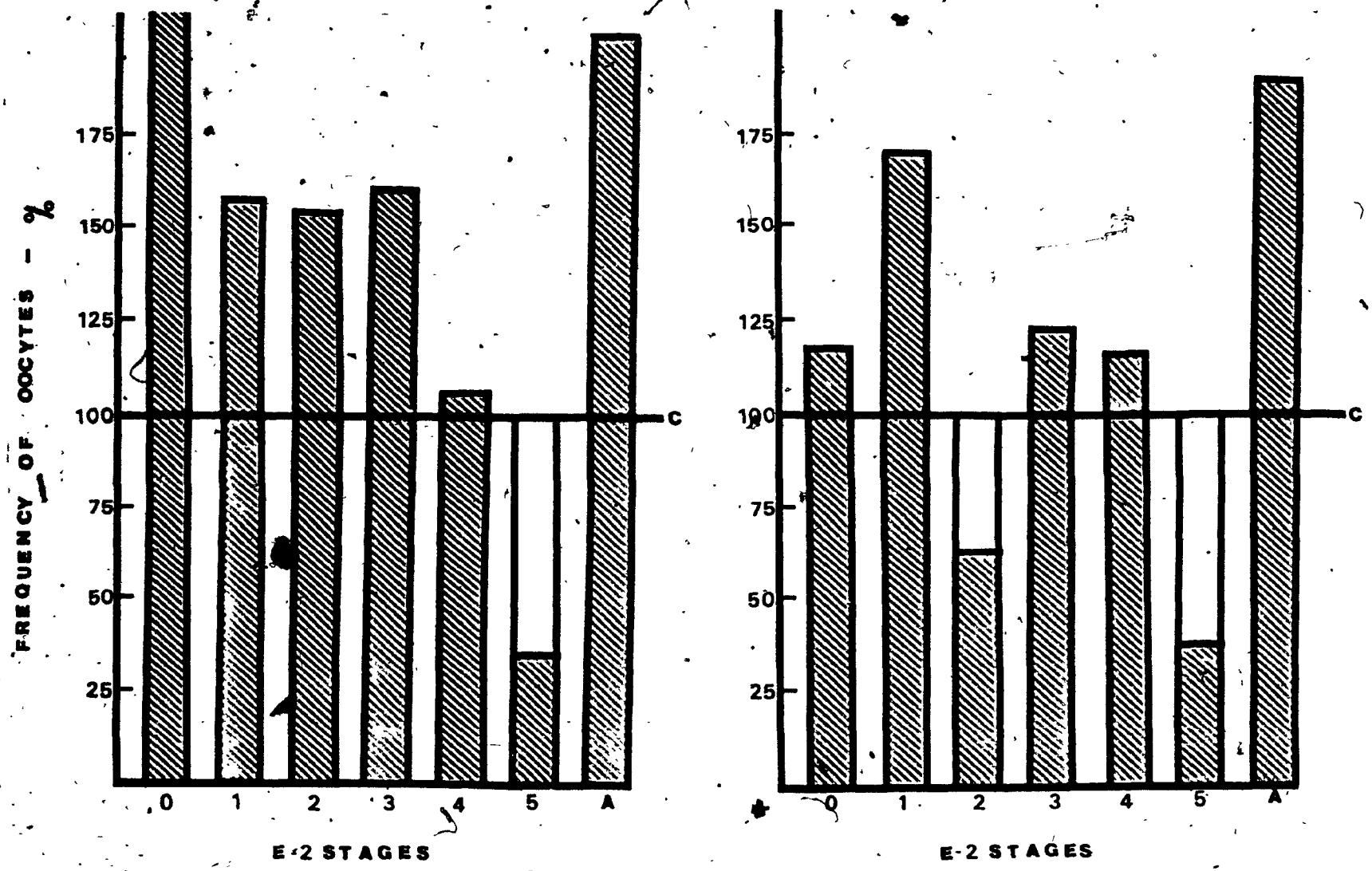

control (C)
0.01 mg/l HCN
FREQUENCY OF OOCYTES — %
175
150
125
100
75
50
25
C
0 1 2 3 4 5 A
E-1 STAGES
control (C)
0.02 mg/l HCN
175
150
125
100
75
50
25
C
0 1 2 3 4 5 A
E-1 STAGES

Figure 50b. Histograms showing the activity of oocyte developmental stages and (A) atretic follicles in control and cyanide-treated fish during Experiment 2.


FREQUENCY OF OOCYTES - %
175
150
125
100
75
50
25
C
0
1
2
3
4
5
A
E-2 STAGES
175
150
125
100
75
50
25
C
0
1
2
3
4
5
A
E-2 STAGES

Experiment Number	Stages	ACTIVITY-% FREQUENCY Tank 0.01 mg/l HCN	Tank 0.02 mg/l HCN
1	0	105.44	145.60
	1	92.77	124.41
	2	126.98	144.82
	3	121.50	141.67
	4	101.87	96.26
	5	15.32	7.21
	A	88.73	76.73
2	0	253.82	117.36
	1	158.29	171.43
	2	153.83	63.64
	3	160.18	122.62
	4	106.12	116.91
	5	35.38	38.46
	A	201.78	188.89

Table 7. Index of activity in oocyte stages as percentage frequency. Controls are indicated as 100% activity per stage. Comparison of cyanide-exposed ovaries for both experiments.

a significant stimulation of atresia. Significant blocks at stages 0,3,5 and atresia are demonstrated at 0.01 mg/l HCN in the same experiment. The decline to 35.38% in stage 5 and the significant atresia activity (201.78%) comprised mainly of late-stage oocytes are evidence of a reduced number of oocytes developing beyond stages 3 and 4.

Observations on Potential Egg Growth

Monthly changes in average oocyte sizes occurred from May through September and were used to study the effects of cyanide on egg maturation. The largest egg diameters were found at all levels of the sectioned ovary. Peripheral zones, however, tended towards smaller-sized oocytes, often irregular in form.

From the arithmetic mean of the 10 largest ova diameters for each ovary, potential growth was analysed between the time intervals of the two experiments performed. Growth within the 15 and 20 days duration of an experiment was also measured. In Table 8., the mean growth over spring (Day 20) to summer (Day 20) controls is a significant 70 microns. The difference between control Day 0 and Day 20 showed approximately 75% significance in both experiments.

Experimental Tank (mg/l HCN)	Sample Size (n)	Mean Major Axis (/1m)	Standard Error (±)	't' value Toxicant Day 20 / Control Day 20
E_1 - 0.00 - Day 0	5	223.13	24.41	0.197
E_1 - 0.00 - Day 20	9	228.00	11.85	
E_1 - 0.01 - Day 20	12	224.45	10.86	0.219
E_1 - 0.02 - Day 20	12	205.59	8.54	1.766 *
E_2 - 0.00 - Day 0	9	276.11	13.38	1.020
E_2 - 0.00 - Day 20	8	298.48	17.69	
E_2 - 0.01 - Day 20	13	265.21	10.28	1.755 *
E_2 - 0.02 - Day 20	8	275.30	12.61	1.067

* $0.05 > p > 0.02$

Note Day 20 is combined with Day 15 plus Day 20 values. E_1= Experiment 1; E_2= Experiment 2. Summer growth between experiments 't' value is significant at 6.396.

Table 8. Maximum growth of sampled ova is represented as mean major axis of the 10 largest oocytes per individual. Potential growth is shown as the difference over the 20 day exposure period.

No valid difference occurred from Day 15 to 20 in either experiment, and the values were accordingly and consistently combined.

The potential growth rates of toxified oocytes were compared to control Day 15 to 20 combined data. Table 8. indicates a significant difference in the mean major diameters in Experiment 1. after 0.02 mg/l HCN exposure. The mean major diameter was significantly smaller than the controls only at 0.01 mg/l HCN in Experiment 2. The frequency histograms of diameter ranges in Figure 51. and 52. reveal the absence of the largest oocytes. In Experiment 1., there is 0% frequency of oocyte diameters greater than 300 microns after 0.02 mg/l HCN exposure (Figure 51.). Similarly, Figure 52. demonstrates 0% frequency of diameter ranges beyond 350 microns for both cyanide concentrations.

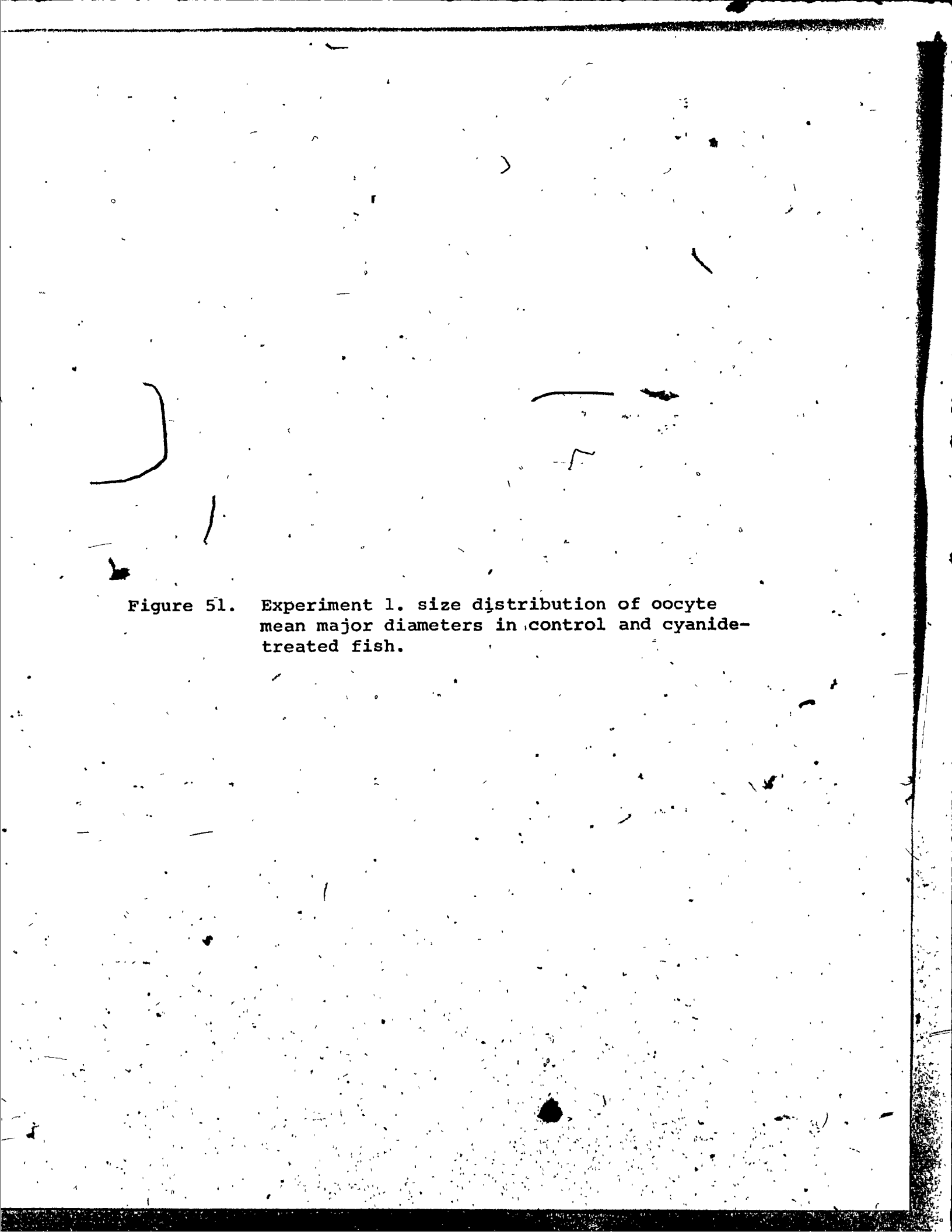


Figure 51. Experiment 1. size distribution of oocyte mean major diameters in control and cyanide-treated fish.

Figure 52. Experiment 2. size distribution of oocyte mean major diameters in control and cyanide-treated fish.

DISCUSSION

Physiological Effects

Significance of Observations in Normal Oocyte Maturation

Lagler et al (1962) have stated that female Salmo gairdneri attain sexual maturity for the first time from the ages 2-5 years or body lengths ranging from 7.5 - 30 cm or more. The young females obtained for these early and late summer experiments measured between 12-16 cm. Histological examination of these ovaries revealed developing ova undergoing maturation for the forthcoming spring spawn.

It was evident from this study that although rainbow trout are annual breeders, the ova are not synchronized at one developmental stage throughout the growing season. Several egg stages were present at any given sampling time which suggests that ripe eggs are shed on more than one occasion. A high variation in the frequency of stages and the degree of maturation among individuals was common. Similar variations were described in the yellow perch, Perca flavescens by Malservisi and Magnin (1968) who indicate that such variance may be anticipated in most fish of temperate waters. Scott (1962) observed wide differences in final egg size for wild populations of

rainbow trout and attributed the differences to intraspecific competition.

Classification of the stages of egg maturation in the Salmo gairdneri studied, was complicated by oocytes of seemingly comparable size displaying a variation in morphological organization. Similar findings have been previously reported for rainbow trout (Beams and Kessel, 1973). Considerable overlap of definitive stage criteria, especially in rapidly proliferating stages 2-5, was visible throughout this study. These included the rates of Balbiani body fragmentation and disappearance, as well as frequently simultaneous deposition of primary and secondary yolk phases which were sometimes common to several oocyte size classes. This indicates a wide variation in the consecutive developmental stages with respect to time among oocytes and expresses varying metabolic rates of activity of these sequences. Large class intervals assigned to egg sizes reduced the overlap and facilitated recognition of true lag effects due to cyanide.

As evidenced from Tables 2. and 3., oocytes undergo a primary growth phase marked by an increase in volume of both cytoplasm and nucleus. The extended "lampbrush" chromatin observed during this period has been attributed to the synthetic activity of genes by previous authors (Beams, 1964).

Multiplication of the nucleoli corresponding with cell growth discerned in this investigation, along with their migration to the preferential nuclear boundary, reflect stimulation of RNA production.

The presence of a prominent Balbiani body, as previously reported in rainbow trout (Beams and Kessel, 1973), was confirmed in this study. Its duplex structure consisting of a non-basophilic, idosome core and densely basophilic, outer pallial substance was clearly evident in oocytes classified at developmental stages 2 and 3. The deeply basophilic layer suggests an endoplasmic reticulum rich in RNA and reflects a period of active protein synthesis (Kemp, 1956).

Guraya (1965) showed histochemically that the Balbiani body of Channa maruleus, a teleost, was partially composed of RNA and associated its production with that of proteins and fatty yolk formation. In more recent studies (Beams and Kessel, 1973), the affinity of the pallial layer for basophilic dyes and its subsequent destruction by hot trichloracetic acid, clearly demonstrate RNA and protein nature. Enzyme studies performed by Livni (1971) on three teleosts demonstrated the Balbiani body to contain succinate dehydrogenase (aerobic), glucose - 6 - phosphate, dehydrogenase (anaerobic) and ∝-glycerophosphate dehydrogenase (anaerobic).

Classified stage 3 and early 4 oocytes displayed accumulations of positive periodic acid-Schiff granules immediately outside the nucleus and radiating into the surrounding ooplasm. Identical granules have been considered glycogen in composition by Beams and Kessel (1973).

As seen in Table 3., the secondary growth phase during stages 4 and 5, is characterized by an exchange of products as the large complement of yolk is incorporated into the extensive mass of cytoplasm.

The results of the present study suggest that the trout oocyte becomes filled with yolk resulting from the action of its own organelles and via selective micropinocytosis with the follicular cells.

Conflicting views of proteid yolk formation have resulted among investigators working on different species of developing eggs. Yolk may be formed either from the oocyte organelles (intracellular), or may be obtained via the follicular cells (extracellular) previously manufactured outside the ovary, or from a combination of both methods. Four patterns have been classified (Wolfe, 1972, p.458-461): Yolk bodies may be formed directly from the mitochondria as described for amphibians . Secondly, yolk may arise by pinocytosis whereby the pinched-off vesicles contain extracellular yolk materials from follicular cells, such as in the case of lampreys. Yolk formation through direct connection with the rough and smooth endoplasmic reticulum has been documented in crayfish oocytes and also from vesicles arising near the elements of endoplasmic reticulum and Golgi as for example in the frog genus Rana. Beams and Kessel, (1973) have demonstrated an extensively developed system of

endoplasmic reticulum having numerous cisternae granules present in the early oocytes of rainbow trout. Their suggested purpose of this system is the elaboration of a specific type of yolk granule.

The initiation of yolk in control rainbow trout was found to begin concurrently with the Balbiani fragmentation (Table 3.). Frothy oil droplets of fatty yolk occupied most of the remaining cytoplasm in early stages 2 and 3. Carbohydrate-bound primary and secondary yolk appeared to be established next within the expanded cytoplasm. Yamamoto and Oota (1967) found mature yolk globules to be randomly scattered in zebrafish eggs. Contrary to their findings, deposition of yolk in rainbow trout within the present study, indicated a repetitive organization. This was marked by a distinct band of yolk first appearing in restricted regions circumscribing the nucleus and secondarily at the periphery of the cytoplasm of the oocyte. Tiny vesicles of primary yolk first occurred in these band formations and spread centrally. When the cytoplasm was almost entirely filled, acidophilic secondary yolk vacuoles occupied the identical, former band regions. Again, these migrated across the ooplasm to unit with previously laid down primary yolk. The final

product by stage 5 was characterized by the merged yolk globules. Gene amplification and the yolk band about the nuclear membrane indicate active synthesis of specific yolk prescribed by the nucleus, and its associated organelles (Golgi apparatus and endoplasmic reticulum). Electron microscopical studies by Beams and Kessel, (1973) have shown passage of granules into the cytoplasm through nuclear pores.

The electron micrographs from this study show evidence of interdigitating microvilli between the inner (epithelial) follicular cell and the ooplasm proper in late stage oocytes. This indicates a secondary site of active yolk synthesis. The increased surface area suggests passage of materials, presumably raw nutrients from follicular cells, into the oocyte cytoplasm. Peripheral yolk band formation observed in the ooplasm adjacent to this region may signify the receiving area of a different and specific yolk. Eventually both yolk types blend into secondary globules. The peripheral band may also function as a supportive structure to prevent collapse of the distal cytoplasm.

A similar description of peripherally banded yolk vacuoles and globules has been reported by Rajalakshmi (1966) Gobius giuris. Braekevelt and McMillan (1967) expressed the same peripheral yolk deposition in brook stickleback. However the formation of yolk in restricted regions circumscribing the nucleus and the repetitive nature of primary and secondary yolk deposition patterns as reported in the present study has not been previously described.

Atresia

Follicular atresia is the natural means within the ovary to eliminate oocytes. Although it is fundamentally the same in all teleosts (Sathyanesan, 1963) it may be present in several forms such as: pre and post-ovulatory, being either hypertrophic (involving phagocytes) or nonhypertrophic.

During the spring and summer experiments, pre-ovulatory, hypertrophic atresia was the most frequent form of degeneration encountered. Normally the process was seen to begin with disintegration of the cytoplasm followed by follicular cell and membrane involution. A large population of phagocytes sometimes containing yolk granules was present.

These findings indicate yolk resorption by phagocytosis where enzymes lyse the cell and the dissolved yolk is subsequently recycled.

The onset of atresia was recognized in all stages of oocyte development, in agreement with Hoar (1966). Stages 3-5 demonstrated the highest percent occurence. Earlier atretic oocytes (stages 0-2) were rarely identified but the process takes place rapidly due to the scant amount of yolk present for resorption.

The highest incidence of atresia was located along the lateral and posterior peripheries of the ovary which may reflect a selective force which protects the innermost oocytes.

Reduction in numbers of oocytes is usually completed early in the secondary growth phase (Henderson, 1963). The control rainbow trout confirmed this finding. With summer growth, the greatest frequency of oocytes shifted to stages 4 and 5 and this was accompanied by a significant decline in control incidence of atretic follicles.

Test fish held at either 0.01 or 0.02 mg/l HCN displayed significantly increased frequencies of atresia in Experiment 2. and were composed mainly of stages 3 and 4. These results suggest that the secondary growth period between stages 3 and 4 is normally more susceptible to atretic degeneration and it is enhanced by cyanide in the water.

The sequence of events involved in egg resorption and removal may stem from a variety of sources. Ball (1960, p. 105-137) related increased atresia in rainbow trout exclusively to restricted experimental diet intake. Similarly, regressed egg numbers in natural populations coincided with decreased stomach content volumes. In this study, optimum feeding may account for the reduced control atresia corresponding to the later summer months' experiment. Therefore it appears that the significant increases in atresia observed in treated ovaries reflects the stimulated effect of cyanide on egg degeneration.

Pathology

Numerous abnormalities of individual oocytes observed within the treated ovaries indicate permanent damage associated with prolonged cyanide treatment.

A tolerance variation was obvious since individuals were not equally affected under the same concentration. In both experiments, a unique morphological aberration within the cytoplasm was confined mainly to oocytes exposed at 0.01 mg/l HCN and was detected in all stages of oocyte development. Deep furrow formations were associated with an infiltration of macrophages. Unlike the phagocytic invasion involved in normal atretic breakdown of oocytes (Braekevelt and McMillan, 1967; Lewis and McMillan, 1965), the rapid surface attack of macrophages recorded in this study reflects a reaction of the reserve defence mechanism of ovaries under cyanide contamination. Bloom and Fawcett (1975) have attributed this mobilization of normally sessile macrophages to stimulation under inflammatory conditions. Evidence that the furrows are consistently and almost exclusively limited to the lowest concentration suggests that 0.01 mg/l HCN is a critical level whereby the ovary attempts to cope with the stress. Selected oocytes may be eliminated to conserve energy which is in turn channeled to completion of other oocytes.

Lesser alterations revealed in treated ovaries included either thickening of the zona pellucida or loss of cell contour and the subsequent production of ellipsoidal oocytes. A low incidence of the ellipsoidal phenomenon was restricted to the peripheral regions of the control ovary whereas at 0.02 mg/l HCN the ovaries revealed ellipsoids throughout the gonad. An intensification of these conditions also increased with increasing cyanide concentration. The enlarged ellipsoidal oocytes were necessarily classified into stage 4 or 5 because of size but were neither atretic nor revealed any furrow formations. The cytoplasm, however, was notably retarded in comparison with controls. This ellipsoidal condition explains the apparent lack of significant differences in the stage frequency data at the 0.02 mg/l HCN concentration in the second experiment. The occurrence of the ellipsoidal oocytes in controls (although rare), implies a common stressing factor in control and treated fish which is enhanced upon addition of cyanide.

Although the mechanism of action is obscure, these distortions may perhaps be caused by minimal inhibition or delay of products essential to cytoplasmic maturation and maintenance of tensile strength in the membranes.

It is of interest that evidence from atresia, furrows and ellipsoids all suggest a similar mode of protection whereby the ovary sacrifices the outermost oocytes in order to compensate internal oocytes. The frequency of atresia was highest on the ovarian peripheries and especially at the posterior tip of the gonad. Likewise, furrow formations and extreme ellipsoids first populated the peripheries and developed inwards.

Some relationship may exist between the internal position of the oocytes and blood supply in the functioning of this protective mechanism.

It is clearly evident from the morphological results that the metabolic rates in cyanide-treated ovaries have been significantly retarded. After 20 days exposure, this was expressed in affected oocytes as an obvious lag in the cytoplasmic products including the Balbiani body, PAS-positive granules or secondary yolk vacuoles.

The arrested Balbiani fragments observed in the cytoplasm reflects a probable cyanide interference within a biochemical mechanism. Livni (1971) has previously identified in the Balbiani body of several teleosts, concentrations of succinate dehydrogenase. This enzyme is involved in catalyzing part of the carbohydrate (aerobic) pathway of Krebs tricarboxylic acid cycle. The succinate dehydrogenase reaction converts succinate to fumarate (Lehninger, 1970, P. 160) and is irreversibly inhibited by cyanide (Keilin and King, 1960). This indicates that inhibition of succinate dehydrogenase may be responsible for the non-dispersion of Balbiani fragments. Since the produced enzyme is inactivated, build-ups of intermediate substrates of the pathway remain in the cytoplasm. As a result the Balbiani cannot continue in protein deposition. The morphological result is expressed by the basophilic patchwork which may represent an excess or unusuable forms of amino acids. These would normally be deaminated and recycled into Krebs cycle for production of compounds with high phosphate transfer potential.

Other alterations in the distribution of brilliantly stained PAS granules throughout stages 3-5 occurred in treated ovaries. The granule aggregates outside the nuclear membrane in stages 3 and early 4 ooctyes lacked the normal dispersion into the cytoplasm. In late stage 4 and 5, these granules formed clumped associations with the secondary yolk vacuoles and failed to disperse into the cytoplasmic band formations. The above observations suggest blocked mucopolysaccharide activity within the oocyte and hence an interruption in the pathway of carbohydrate-bound yolk deposition.

The effects observed may in part be related to a second organ such as the liver, which is known to manufacture intermediary yolk products in several animals (Droller and Roth, 1975; Schjeide et al, 1963). These are possibly transferred to the ooctye via follicular cell microvilli. Dixon (1975), using concentrations of cyanide as low as those of this study showed some degree of hepatocyte degeneration in all rainbow trout specimens examined. Blocked mucopolysaccharide activity of the present investigation may illustrate a secondary effect on yolk synthesis brought about by impaired liver function.

The mitochondria are the ultimate source of energy for all work performed within an organism. Many stimuli which may lead to cell injury or death begin in these organelles. Fawcett (1969, P. 82) states that normal mitochondria within relatively inactive cells tend towards few cristae and a simple internal organization. Cells actively engaged in synthesis have a large number of mitochondria containing many cristae. A review by Rouiller (1960) has shown that the cristae are not stable structures and are capable of shape changes.

Early structural alterations in mitochondria indicating the onset of pathological conditions are a loss of matrix granules and swelling of the mitochondrion. The properties of irreversible damage include formation of dense particles, absence of cristae and disruption of the membranes (Anderson and Scotti, 1972, P. 4-7).

After 20 days cyanide exposure, mitochondria of several late-stage oocytes and follicular cells showed substantial evidence of early and advanced pathological conditions. These changes include swelling of the mitochondria, transformed cristae and flux of matrix granules.

Mitochondrial swelling denotes initial damage within the cell due to a reversible osmotic disorder (Rouiller 1960). Hypertrophy of the double membranes and the interspace as seen in cyanide ovarian tissue indicates a response to the severe swelling. Rouiller (1960) has related vesiculation and loss of cristae with mitochondrial swelling. The same author described swollen mitochondria resulting from a variety of sources such as starvation, tissue fragments immersed in hypertonic, acid or alkaline solutions, etc. However, prolonged swelling following cyanide exposure probably accounted for the ruptured double membranes observed in this investigation and signified organelle death. These findings suggest that the mitochondrion under lengthy duress of ATP depletion is fatally directed towards the equilibrium (steady) state in which the free energy decreases to a minimum within the cell (Lehninger, 1970, P. 293).

The conspicuous matrix granules which are cation binding sites are normally present among the cristae and are believed to regulate the internal ionic environment of the mitochondrion (Bloom and Fawcett, 1975, P. 48). Disappearance of matrix granules at 0.01 mg/l HCN and concentration of dense, similar particles at 0.02 mg/l HCN

may reflect an obscure relationship between cyanide and the transfer of sodium and potassium ions within the mitochondrion.

The observed structural changes in cyanide-treated oocytes are probably related to two key enzymes: cytochrome oxidase and succinate dehydrogenase. The former is involved in electron transfer and oxidative phosphorylation while the latter is a Krebs cycle enzyme associated with the conversion of succinate to fumarate by removal of hydrogen ions and electrons. These two enzymes, associated with the inner mitochondrial membrane (excluding the cristae), are active in the formation of ATP. The greater portion of ATP is derived from the Krebs cycle and although there may be a temporary borrowing of energy from other cycles such as anaerobic glycolysis, these sources would quickly become depleted.

Cyanide has also been shown to inhibit those enzymes which are dependent on Fe^{2+} or Fe^{3+} by forming inactive complexes similar to ferrocyanide or ferricyanide (Lehninger, 1970, p. 162). The iron-porphyrin molecule is the active

center in cytochrome oxidase. Cyanide inhibition strikes specifically at the terminal portion of the respiratory chain from cytochrome a + a3 (oxidase) to oxygen electron transfer (Lehninger, 1970, p. 379). No new ATP is obtained and the dependent cells such as oocytes gradually become depleted of energy resulting in a back-up of waste products such as lactic acid.

Unlike the cytochrome system, succinate dehydrogenase does not contain a heme group but the Fe^{2+} and Fe^{3+} ions also undergo valence changes (Lehninger, p. 352). Succinate dehydrogenase is produced on the inner mitochondria, is linked to the cytochrome system and is blocked by cyanide as previously outlined in this discussion under Balbiani lag.

The energy stress under cyanide is delicately balanced within the organism. It appears that chronic cyanide forces energy to be redirected away from the reproductive activities to maintenance of priority systems. Although cyanide detoxification processes including methemoglobin, thiosulphate and possibly rhodenase exist in fish (Dixon, 1975), once these mechanisms are cyanide-saturated the progressively deteriorating effects may be

expressed in several systems. For example, Bahr (1972) demonstrated that the physiological behavior in fish suffering from cyanide was similar to hypoxia. Both agents produced conditions of seriously reduced heart functioning evidenced in electrocardiograms. HCN first enters the blood system via the gill apparatus. Reduced blood circulation from altered heart output results in a lack of tissue oxygen and a build-up of biproducts such as lactic acid, potassium and carbon dioxide. These result in damaging effects on the nerve cell membranes, altering impulse conduction (Bahr, 1972; Neil, 1957).

Frequency

Not all oocytes exposed to cyanide developed morphological changes. This permitted quantification of the metabolic differences existing in control and apparently normal cyanide oocytes using stage frequency counts. Egg size (major axis diameter) was used in the potential growth surveys. Since it is an indicator of metabolic activity, these results along with the frequency data aided in supporting the pathology findings.

It is important to realize that a combination of cytoplasmic features and diameter ranges determined frequency counts. Diameter size was ultimately used to classify oocytes such as the extreme ellipsoids experienced under 0.02 mg/l HCN treatment in Experiment 2.

Normal-looking oocytes affected by a delay or block in the overall maturation process due to cyanide may account for the shifts in stage frequencies. The stages most affected when compared to controls were stages 3 and 5 as outlined in Tables 6,7. In Experiment 1., ovaries exposed to the highest concentration resulted in a significantly increased stage 3 and decreased stage 5 respectively. The late summer experiment control ovaries demonstrated an anticipated increased frequency shift among late stages 4 and 5 with the increased egg size presumably requiring higher energy levels. At the lowest concentration of Experiment 2., significant increases occurred in stages 0,3 and 5. The 0.02 mg/l HCN concentration revealed significant increase in stage 1 oocytes and reduced stage 5 frequencies. These responses of increased frequency indicate within affected oocytes a delay in passing onto the next stage of

maturation and is reflected in the reduced numbers of oocytes arriving at stage 5. This indicates that over stages 3 to 4 the involvement in protein and early carbohydrate yolk deposition are the highest energy periods of the developmental stages described and are followed in order by stages 5,2,1 and 0.

The apparent lack of significant changes in Experiment 2 at 0.02 mg/l HCN, may be explained by several factors. Extreme ellipsoids previously described were most frequent during this experiment and at the highest concentration. These oocytes did not appear atretic yet remained immature morphologically when compared to control oocytes of equivalent dimensions and were accordingly classified strictly by size into the appropriate range. This classification combined with high individual control variation plus small sample size may have contributed to the seemingly lessened response in stage 3 frequencies.

Potential growth rates measured over the 20-day term confirmed the control frequency data by indications that cyanide-treated oocytes displayed slower growth rates. A similar trend was apparent with significantly lower (mean) maximum growth at 0.02 mg/l HCN in the first experiment

whereas the effective concentration in experiment 2. was at 0.01 mg/l HCN. The frequency of diameter classes corresponding to the last experiment exhibited 0% of oocytes with diameters greater than 400 microns for both test concentrations. These findings indicate that the diameter frequencies is a more sensitive method than that of the mean largest diameters for detecting subtle effects of cyanide on overall oocyte maximum and potential growths.

The above frequency distribution and potential growth analysis on cyanide-treated ovaries are quantitatively consistent with the observed histological changes reported in this study. These results collectively illustrate the inability of the ovary to endure reduced energy supply due to cyanide under prime growth periods.

It appears that as the energy demand within the ovary increases later in the summer season, the consequences become intensified.

Other investigators have associated changes in stage frequencies of oocytes with a variety of environmental changes. Studies by Aczel and Ruby (1975) using similar frequency counting methods revealed reduced ability of flag-

fish oocytes to deposit secondary yolk after exposure to pH levels below 6.7. Retardation of oocyte growth stages 2-5 was also evident and this implied disturbed protein production. Webb et al (1974) found preliminary results indicating increased proportions of atretic eggs in rainbow trout after lengthy exposure to potassium ethyl xanthate. Similarly, Tafanelli and Summerfelt (1975) examined ova stage development in goldfish after cadmium exposure. Groups receiving multiple injections had 0% frequency of occurrence of stages 5-7 and few ova developed beyond stage 4. Lower doses also showed less abundant late-stage oocytes and corresponding increased frequencies of earlier stages.

Ecological Effects

Large quantities of nutrients - carbohydrates, proteins and lipids, must be stored in the eggs for development of the young progeny. These materials are originally acquired by means of feeding. Interference of food availability through unfavourable environmental conditions may reduce body growth and would ultimately affect oocyte development.

Previous investigators (Speyer, 1975; Dixon, 1975) have demonstrated wet weight loss in rainbow trout following chronic exposure to 0.02 mg/l HCN. In Experiment 1. and 2., fish under cyanide stress demonstrated similar findings. This may be in part explained by the observed altered feeding habits. However, Leduc, (1966b) fed cichlids unrestricted amounts of natural diet following exposure for 36 days at cyanide concentrations ranging from 0.008 to 0.10 mg/l HCN. and noted increased uptake with lowered food conversion efficiency. In the first experiment, the final mean wet weights were significantly lower only at 0.02 mg/l HCN compared to controls. No significant difference was revealed after the second experiment. However, the fish in all three tanks of Experiment 2. showed significant weight change over 20 days duration. The separated females data indicates a significant weight loss in Experiment 1. at 0.02 mg/l HCN and at both concentrations in Experiment 2.

Love (1970) estimated that 8% of the total lipid content of female sockeye salmon was transferred to the ovary during egg maturation. The observed experimental 1. and 2. wet weights despite the small sample size, may possibly reflect a greater effect of cyanide upon female rainbow

trout undergoing gonad development.

Morphological changes in the ovary as a consequence of reduced nourishment have also been reported (Scott, 1962). The incidence of atresia within rainbow trout was related to restricted quantities of experimental diet under otherwise normal circumstances. Studies on natural populations indicated that follicular atresia coincided with decreased stomach contents throughout late summer months. The controlled optimum holding conditions may be responsible for the decreased mean frequency of atretic oocytes in Experiment 2. controls. It seems likely that under the competitive stresses of the natural environment, the impact of cyanide could result in intensified effects on the reproductive system of the rainbow trout. Although the 20-day exposure represents a relatively short period of the total developmental term, both concentrations at 0.01 and 0.02 mg/l HCN elicited different and highly effective results brought about by energy reduction.

The various qualitative and quantitative observations of sublethal cyanide have disclosed some of the chronic effects upon the ovary of Salmo gairdneri. These findings

reflect the sensitivity of rainbow trout ovary and in combination point towards possible reduction in the potential numbers of viable eggs available for fertilization and delayed spawning under possibly adverse environmental conditions. Low food supply, predation and unfavourable temperatures may inhibit embryo development. Eggs of spring and fall spawners may be shifted to summer and late fall respectively. Summer egg deposition could be affected by high temperatures and low oxygen availability while cold water temperatures could be detrimental to egg laying in late fall. Gall (1975) has indicated such a possibility in rainbow trout where lowered growth rates of offspring which were spawned late in the season were attributed to cooler water temperatures. Since the inherent timing of each species presumably ensures optimum reproductive success, the delay findings of this study emphasize the seriousness of the cyanide problem. Furthermore, fish held in the laboratory under optimum conditions would probably be less susceptible to the total impact of cyanide. However, within the natural environment, stresses of competition in addition to cyanide may magnify the effects and further contribute to a reduction in fecundity. Environmental changes inflicted artifically

by pollutants such as cyanide could lead to the eventual decline or extinction of this species.

Game fish including rainbow trout are minimally protected by governmental agencies because of their potential economic importance. Other aquatic vertebrate and invertebrate organisms possessing limited commercial value contribute significantly to the food chain and may be similarly vulnerable to the effects of cyanide. These organisms have little assurance of protection and survival with continued cyanide dumping in aquatic ecosystems.

To date, only the Newfoundland Department of Provincial Affairs and Environment assumes responsibility for water courses and limits the discharge of cyanide to 0.025 mg/l. The target levels proposed for Alberta gold mining industry are listed at 0.1 mg/l CN^- with future consideration to allow 1.0 to 5.0 mg/l ferro cyanide. Objectives for cyanide levels outlined a British Columbia report in 1973 aim at 0.10 mg/l total cyanide but presently approve higher levels up to 2.00 mg/l CN (level C). There have also been some international guidelines for the Saint John River Basin, Nova Scotia (1975) concerning cyanide

which recommend no more than 0.02 mg/l for wildlife and aquatic organisms. The United States Environmental Protection Agency (Ann., 1973, pp. 189-190) sets the maximum concentration of cyanide at 0.05 mg/l HCN.

It appears obvious from this study that acceptable levels of hydrocyanic acid in receiving waters must be lower than 0.01 mg/l HCN in order to ensure reproduction and survival of normal populations of sensitive species such as *Salmo gairdneri*.

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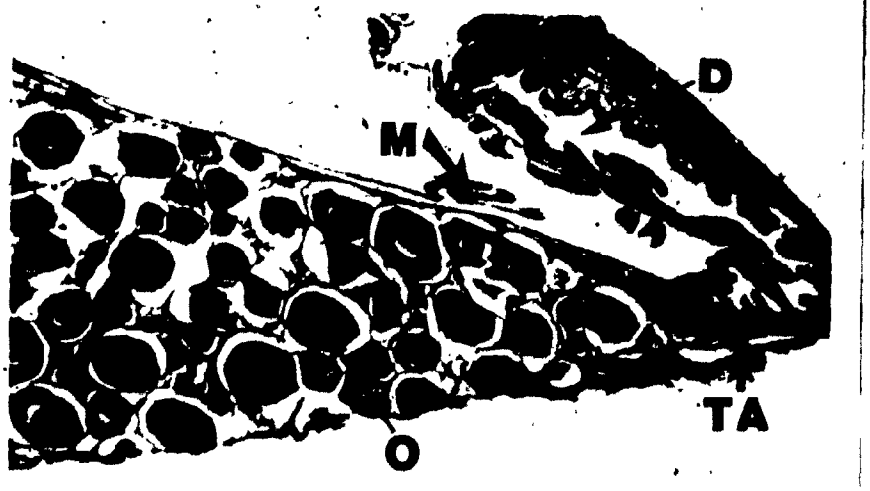
Figure 2. Posterior portion of the ovary (l.s.) showing the duct (D) formed at the genital pore for the release of ripe ova. Oocytes (O) are embedded in the vascular tunica albuginea (TA) which lies beneath the mesovarium (M). (132 X)

Figure 3. Lamellar folds (LF) projecting into the lumen of the ovary. Oocytes at all stages of development are present. (132 X)


D
M
TA
O


LF

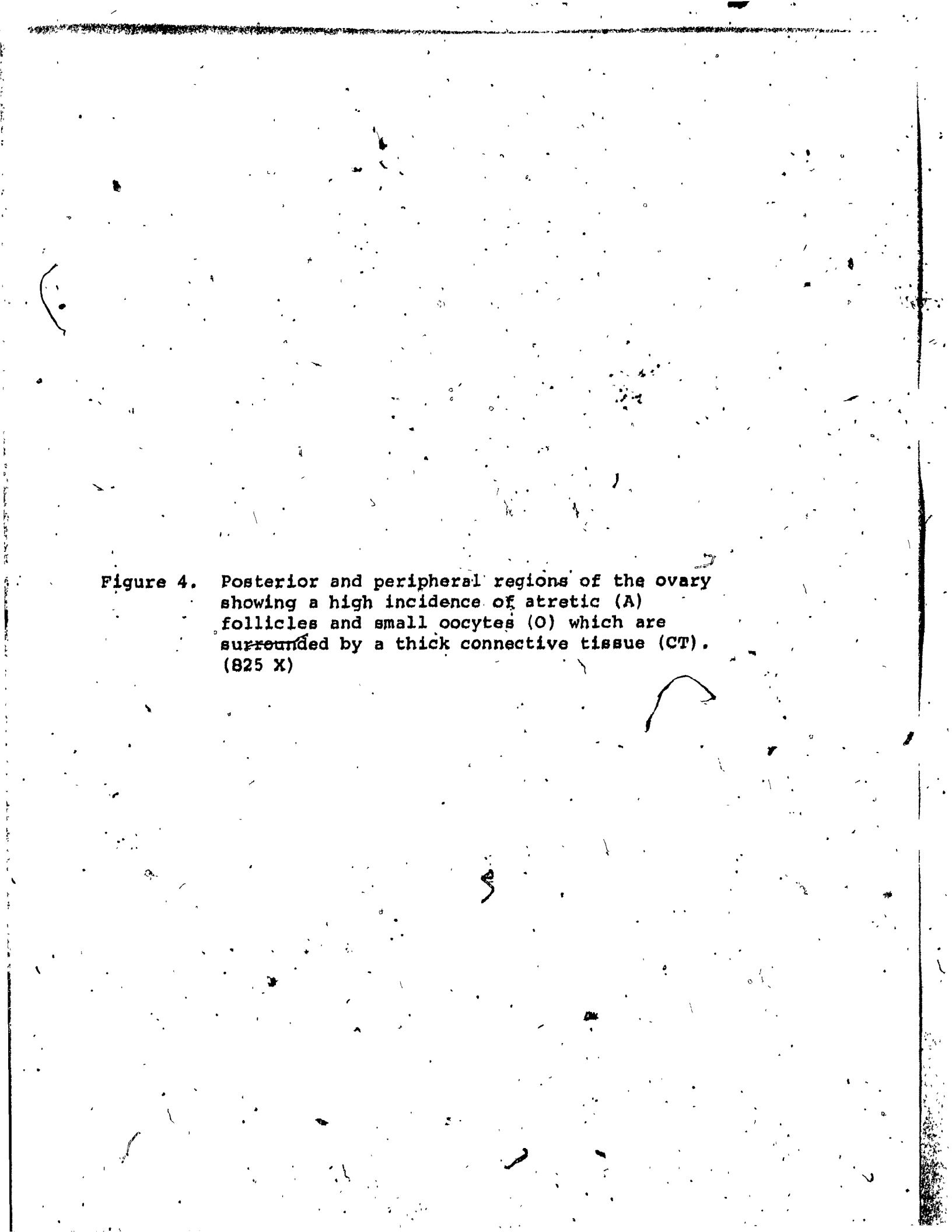


Figure 4. Posterior and peripheral regions of the ovary showing a high incidence of atretic (A) follicles and small oocytes (O) which are surrounded by a thick connective tissue (CT). (825 X)

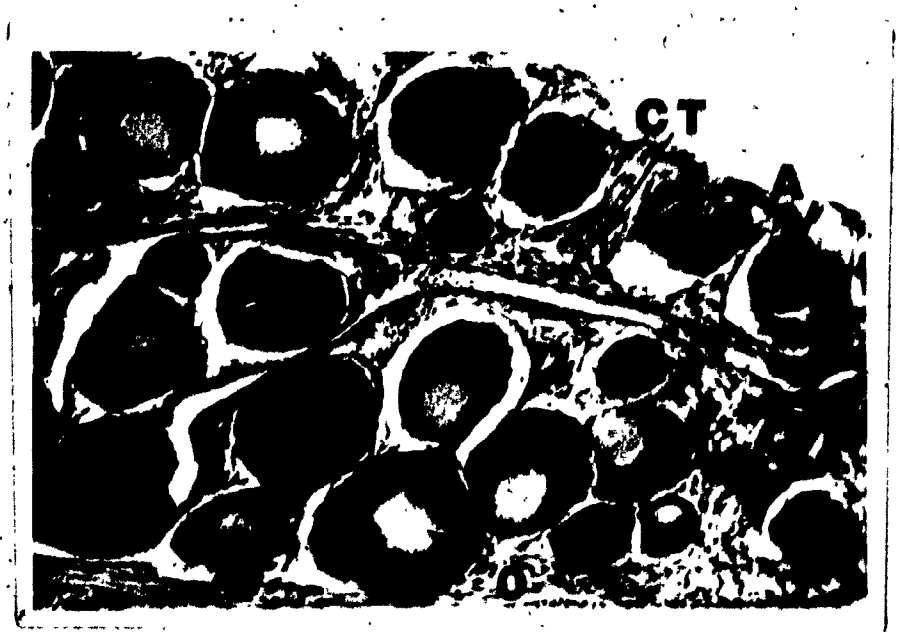

CT

Figure 5. Edge of lamellae (L) illustrating many resting oogonia (RO). The dark nucleus (N) is surrounded by a lightly basophilic rim of cytoplasm having no distinct membrane. (3,300 X)

Figure 6. A pair of recently divided oogonia (DO) in a "nest" (N) association is embedded within the stromal connective tissue (S). A mitotic figure (MF) borders one nucleus. (4,400 X)

Figure 7. Cell nest containing stage I, primary oocytes (PO). The nucleus is larger than those in oogonia, and no nucleoli are present. Investing cells (I) occupy the spaces in the stroma. (3,300 X)

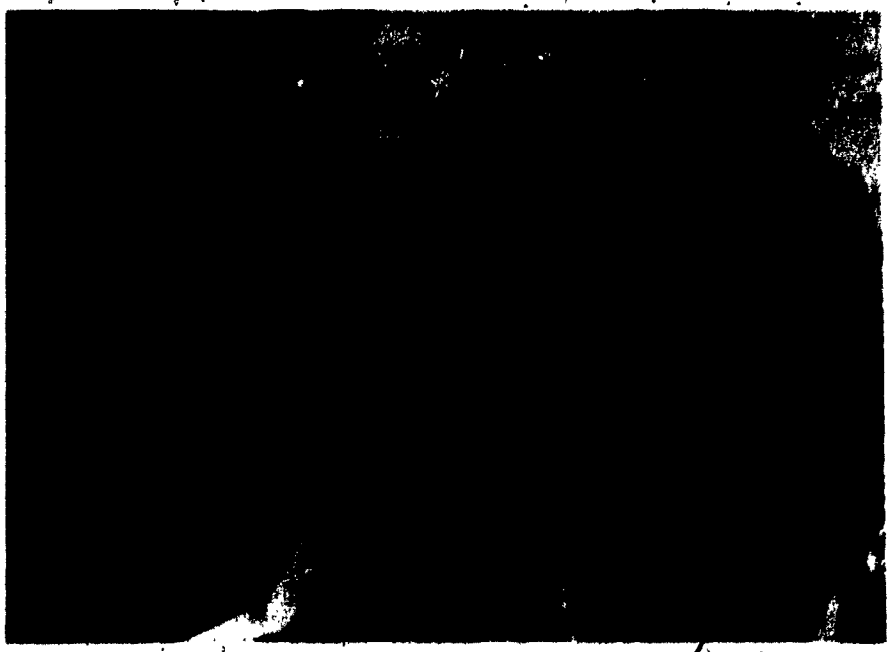
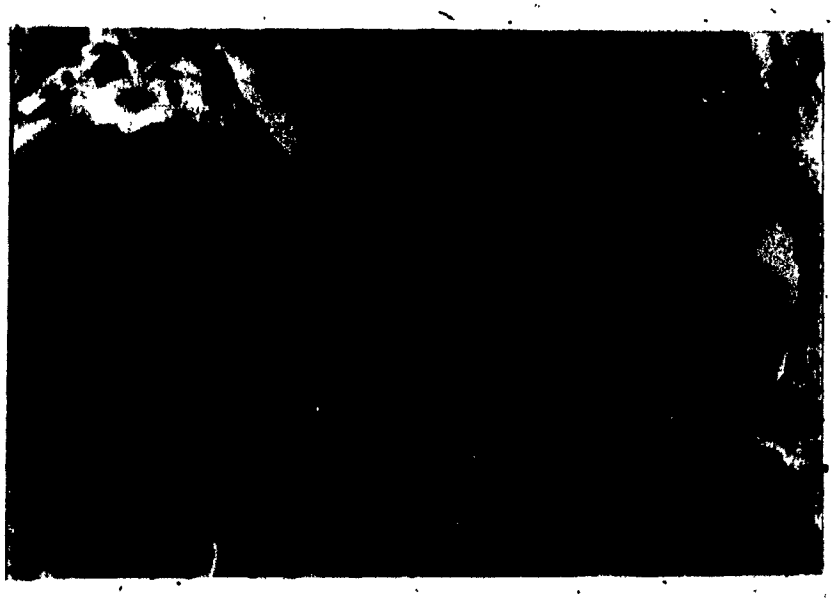


Figure 8. Stage 2 oocyte with juxtanuclear Balbiani body (BB) in earliest form. A central idosome (I) is surrounded by densely stained pallial (P) substance. The nucleus (N) has a distinct membrane and several eccentric nucleoli (Nu). Note the ellipsoidal contour beginning. (1,320 X)

Figure 9. Pallial (P) layer of the Balbiani forming concentric rings in the ooplasm of later stage 2 oocyte. Faint chromatin (C) within the nucleus loses affinity for stains. The investing cells form an incomplete layer of follicle cells (CF). (3,300 X)




P
C
CF

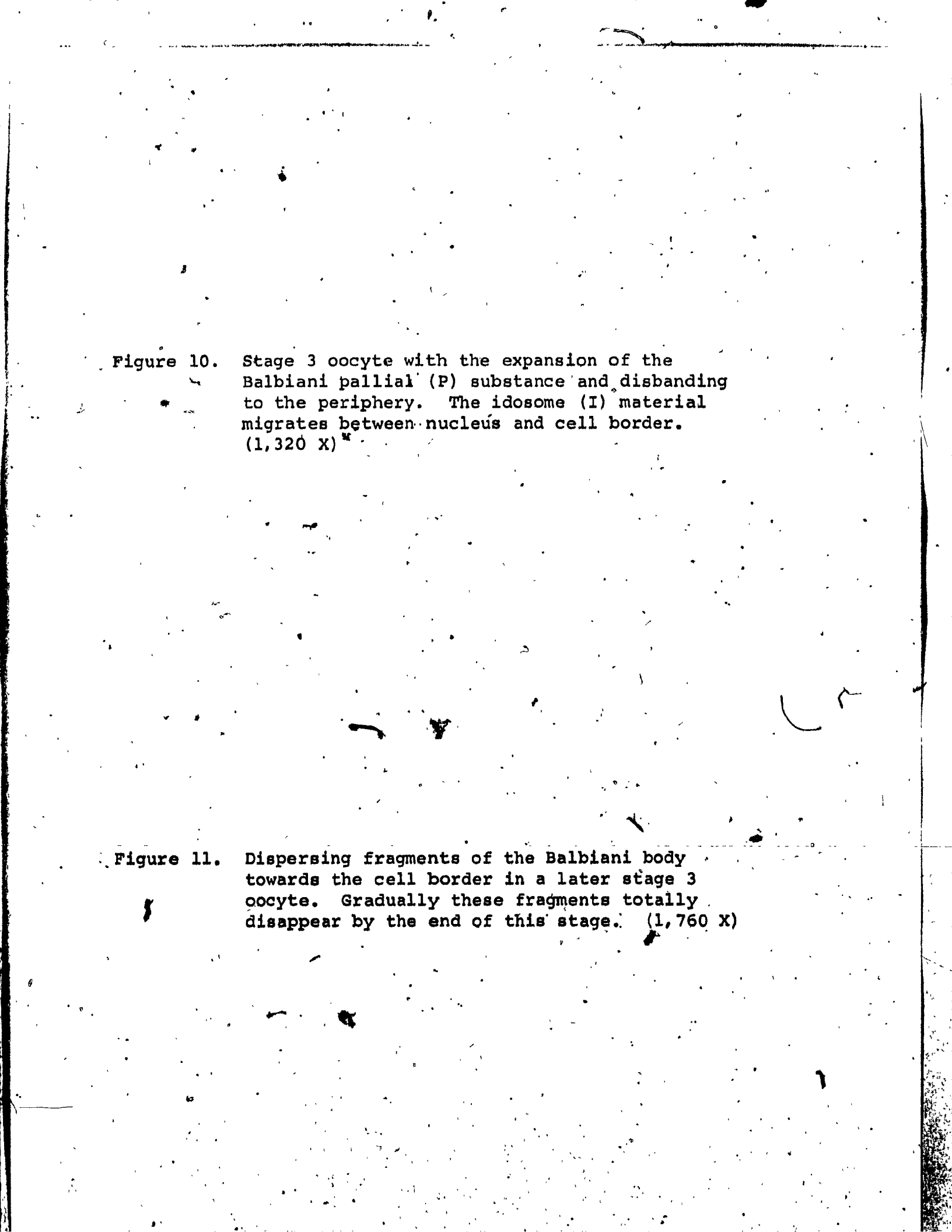


Figure 10. Stage 3 oocyte with the expansion of the Balbiani pallial (P) substance and disbanding to the periphery. The idosome (I) material migrates between nucleus and cell border. (1,320 X)

Figure 11. Dispersing fragments of the Balbiani body towards the cell border in a later stage 3 oocyte. Gradually these fragments totally disappear by the end of this stage. (1,760 X)

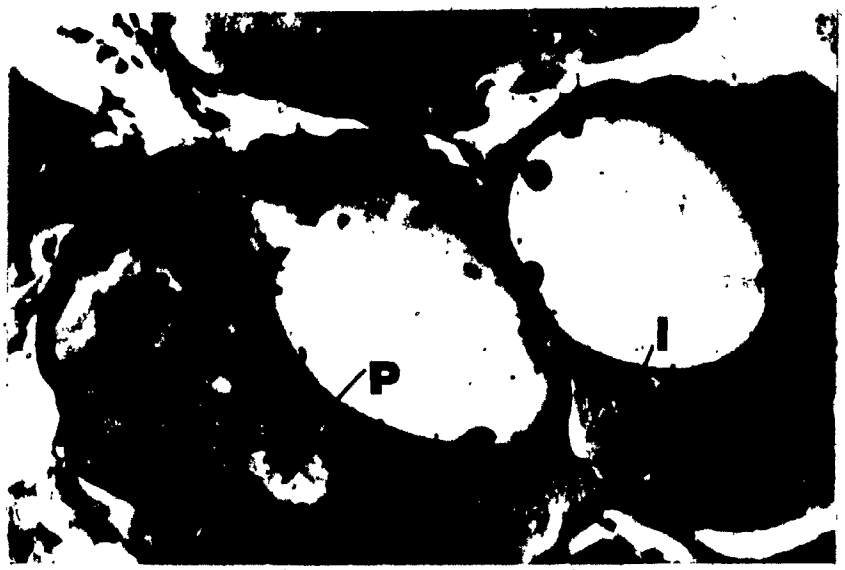
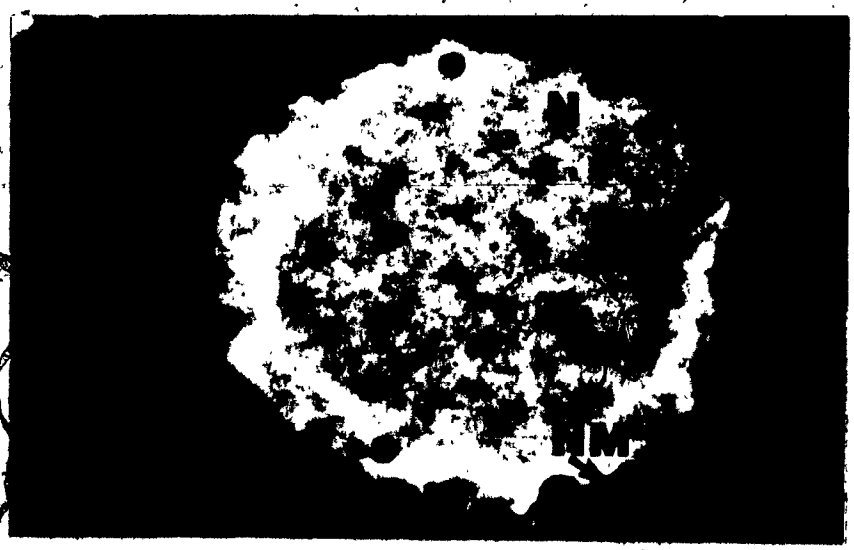

P
I

Figure 12. The periphery ooplasm appears frothy due to droplets of primary yolk ($1^{o}Y$) washed out by ethanol preparation. The egg membrane (EM) and zona pellucida (ZP) ensheath the late stage 3 oocyte. A squamous layer of follicle cells (FC) is completed. (3,300 X)

Figure 13. The centralized nucleus (N) resumes the lampbrush chromatin (LC). Numerous nucleoli (Nu) have migrated to the nuclear periphery. Clusters of PAS-positive granules (Gr) congregate directly outside the nuclear membrane (NM). (3,300 X)


FC


N
M

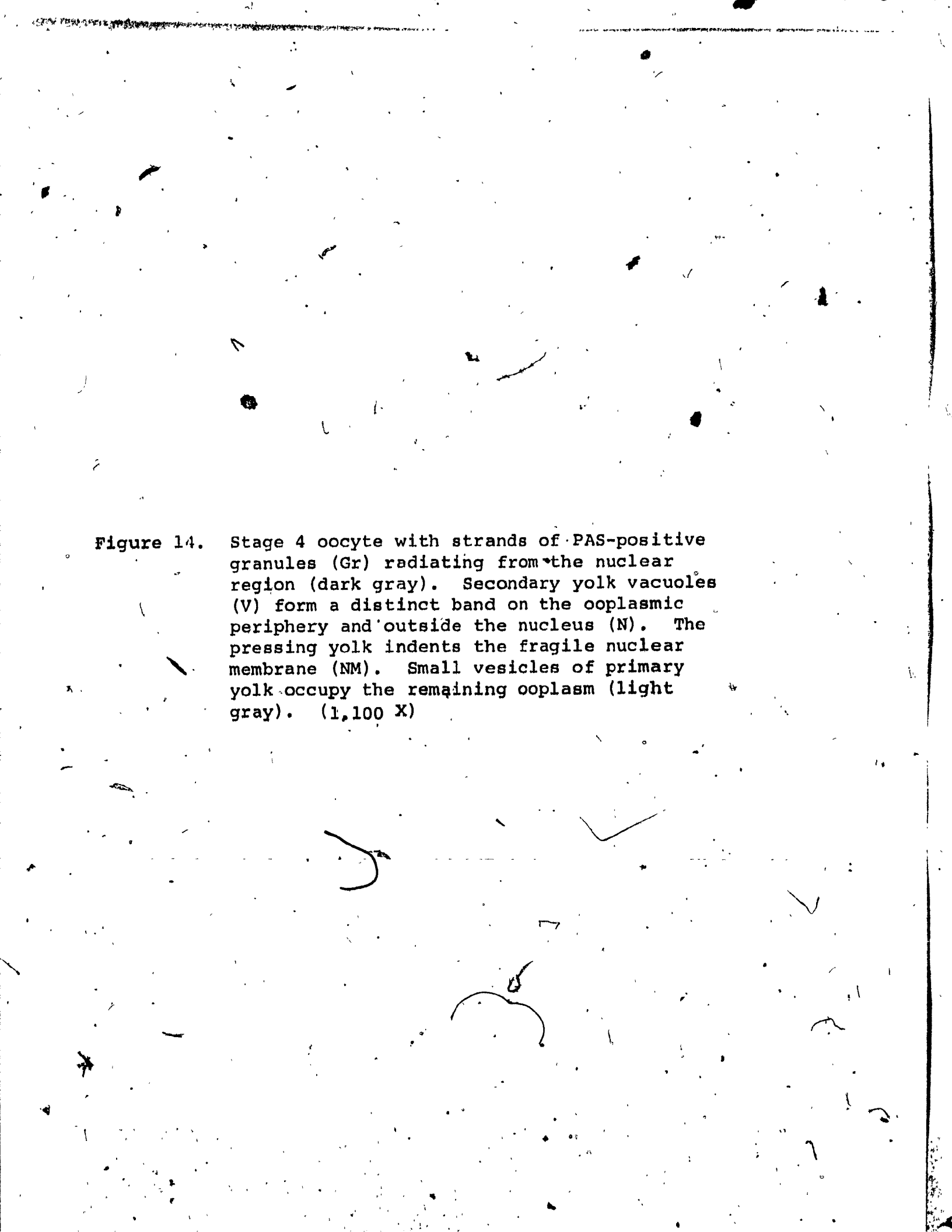


Figure 14. Stage 4 oocyte with strands of PAS-positive granules (Gr) radiating from the nuclear region (dark gray). Secondary yolk vacuoles (V) form a distinct band on the ooplasmic periphery and outside the nucleus (N). The pressing yolk indents the fragile nuclear membrane (NM). Small vesicles of primary yolk occupy the remaining ooplasm (light gray). (1,100 X)

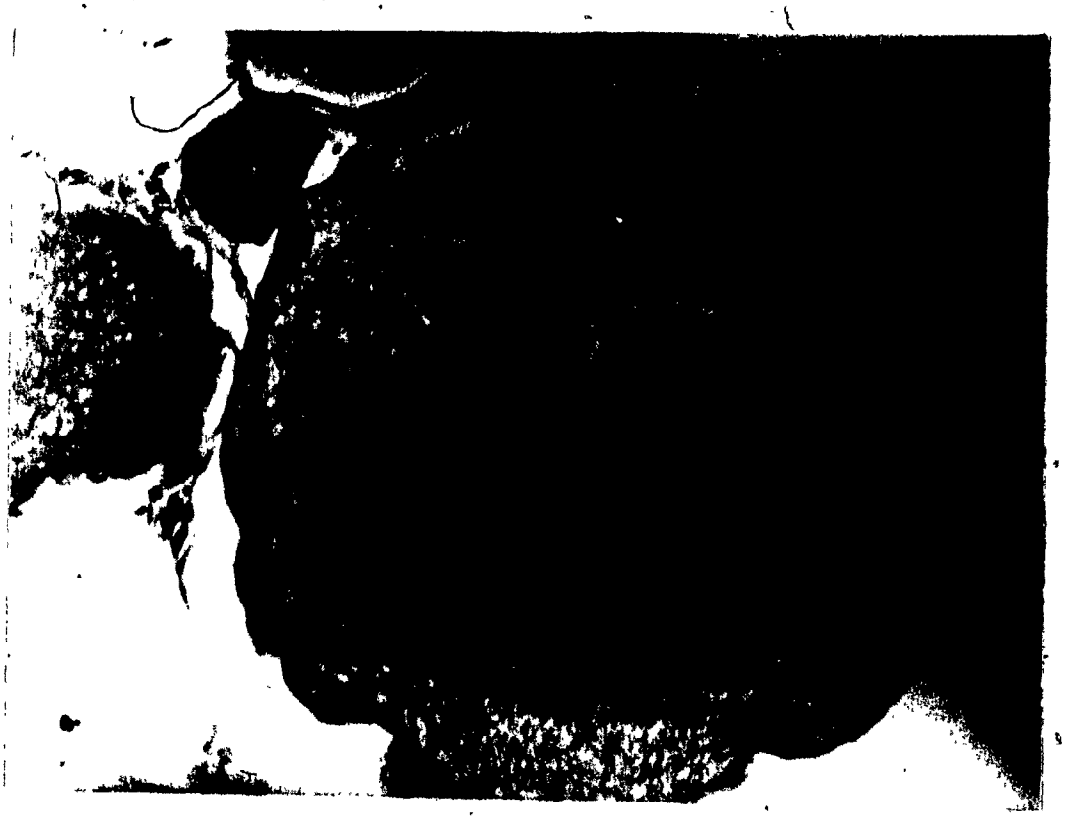


Figure 15. Two stage-4 oocytes demonstraing advanced secondary yolk vacuoles (2^{o}YVa) and fusion with primary yolk vesicles (1^{o}YVe) within the ooplasm. (1,760 X)

Figure 16. Nuclear region revealing zones of combined yolk vacuoles (YV) which press inwards on the nuclear membrane (NuM). Extended lampbrush chromosomes (LC) are present within the nucleus (N). (3,300 X)

Figure 17. Follicular cells consist of an inner, single layer of epithelial cells (FEC) and an outer thecal (T) layer. The zona pellucida (ZP) appears between these two layers. The egg (vitelline) membrane (EM) is closely adherent to the egg surface. (3,900 X)


FEC

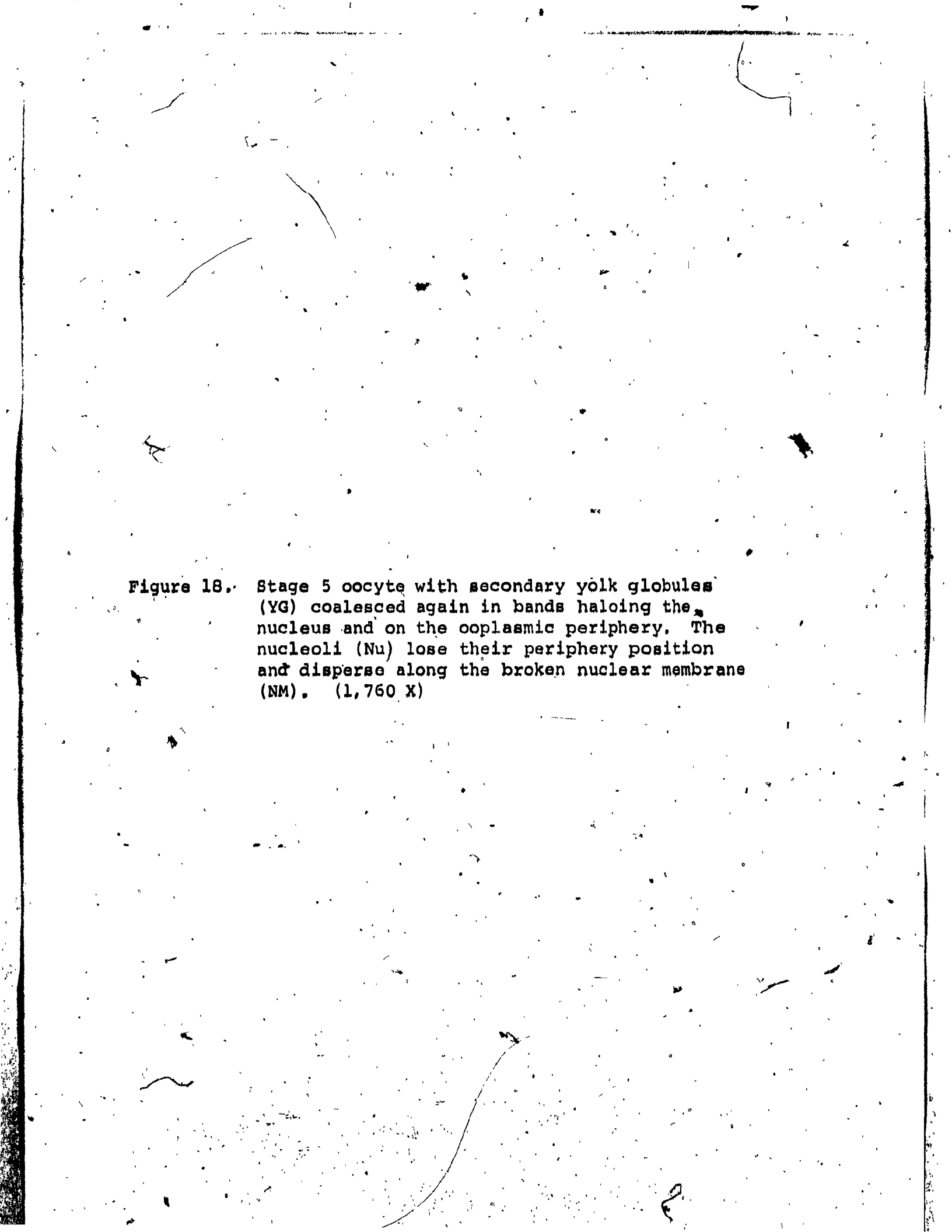


Figure 18. Stage 5 oocyte with secondary yolk globules (YG) coalesced again in bands haloing the nucleus and on the ooplasmic periphery. The nucleoli (Nu) lose their periphery position and disperse along the broken nuclear membrane (NM). (1,760 X)

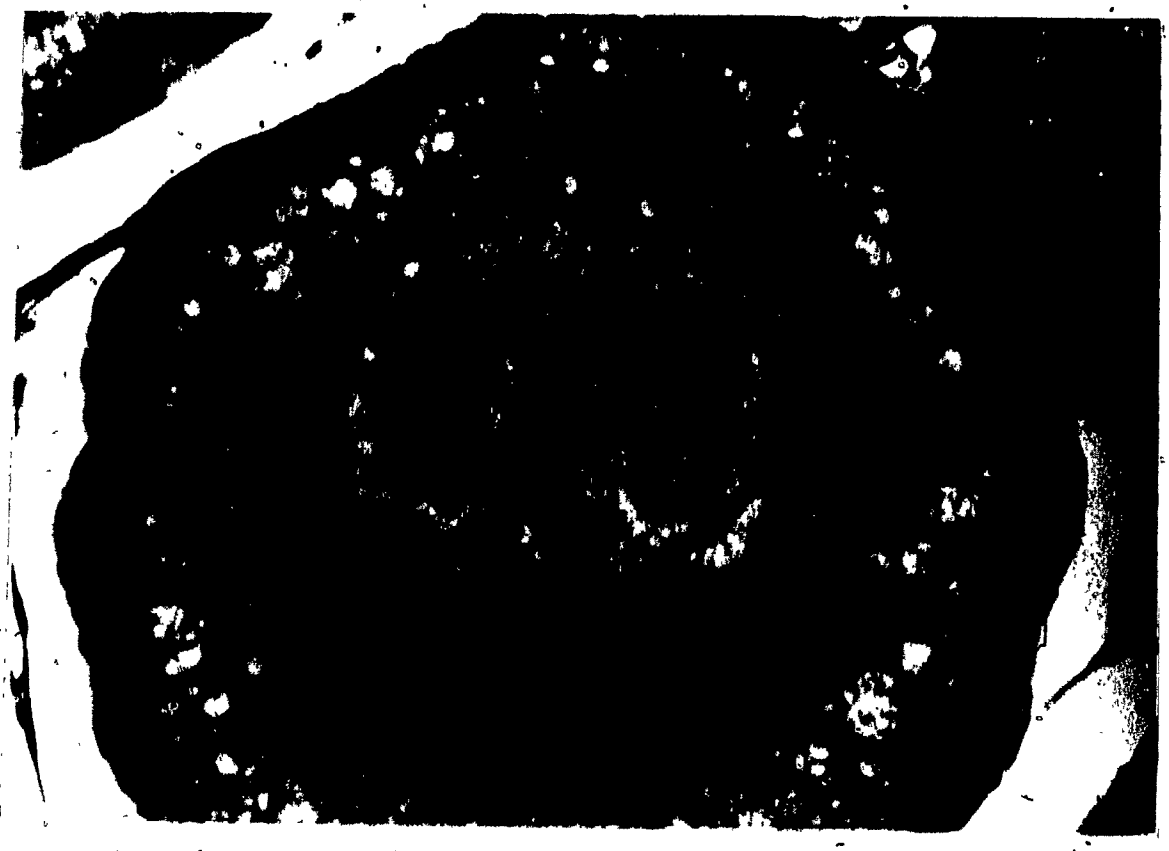


Figure 19. Initial state of pre-ovulatory atresia within a stage 4 oocyte. A ridge of yolk vacuoles (YV) at the cell border is undergoing degeneration. (1,320 X)

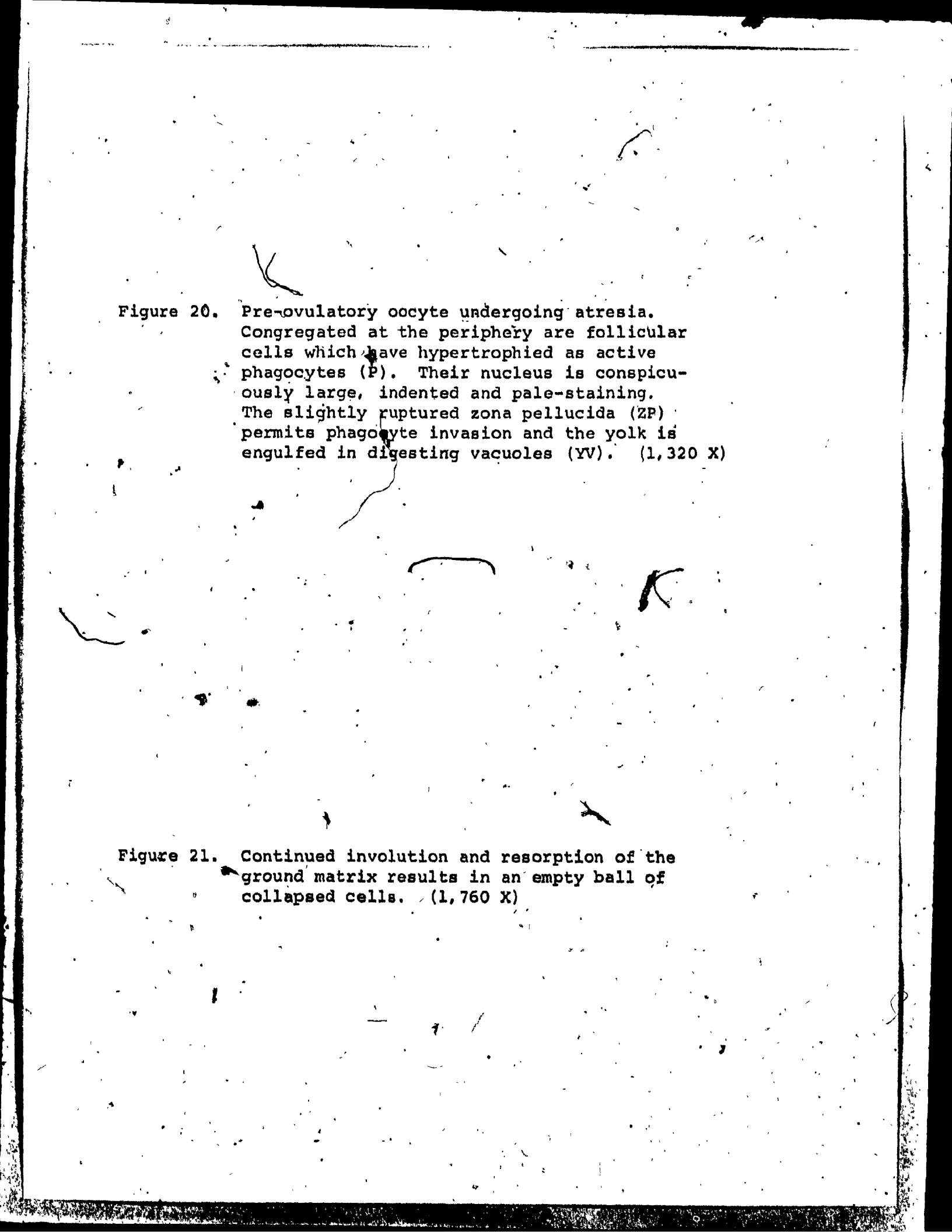
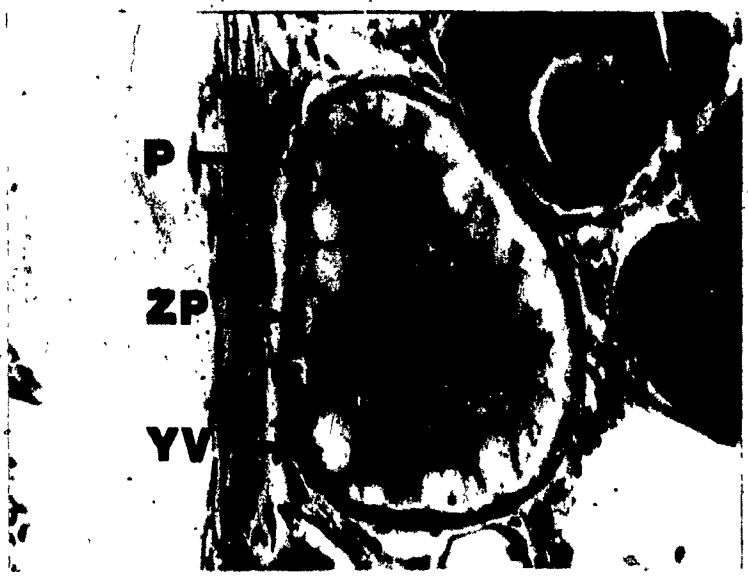


Figure 20. Pre-ovulatory oocyte undergoing atresia. Congregated at the periphery are follicular cells which have hypertrophied as active phagocytes (P). Their nucleus is conspicuously large, indented and pale-staining. The slightly ruptured zona pellucida (ZP) permits phagocyte invasion and the yolk is engulfed in digesting vacuoles (YV). (1,320 X)

Figure 21. Continued involution and resorption of the ground matrix results in an empty ball of collapsed cells. (1,760 X)


P
ZP
YV

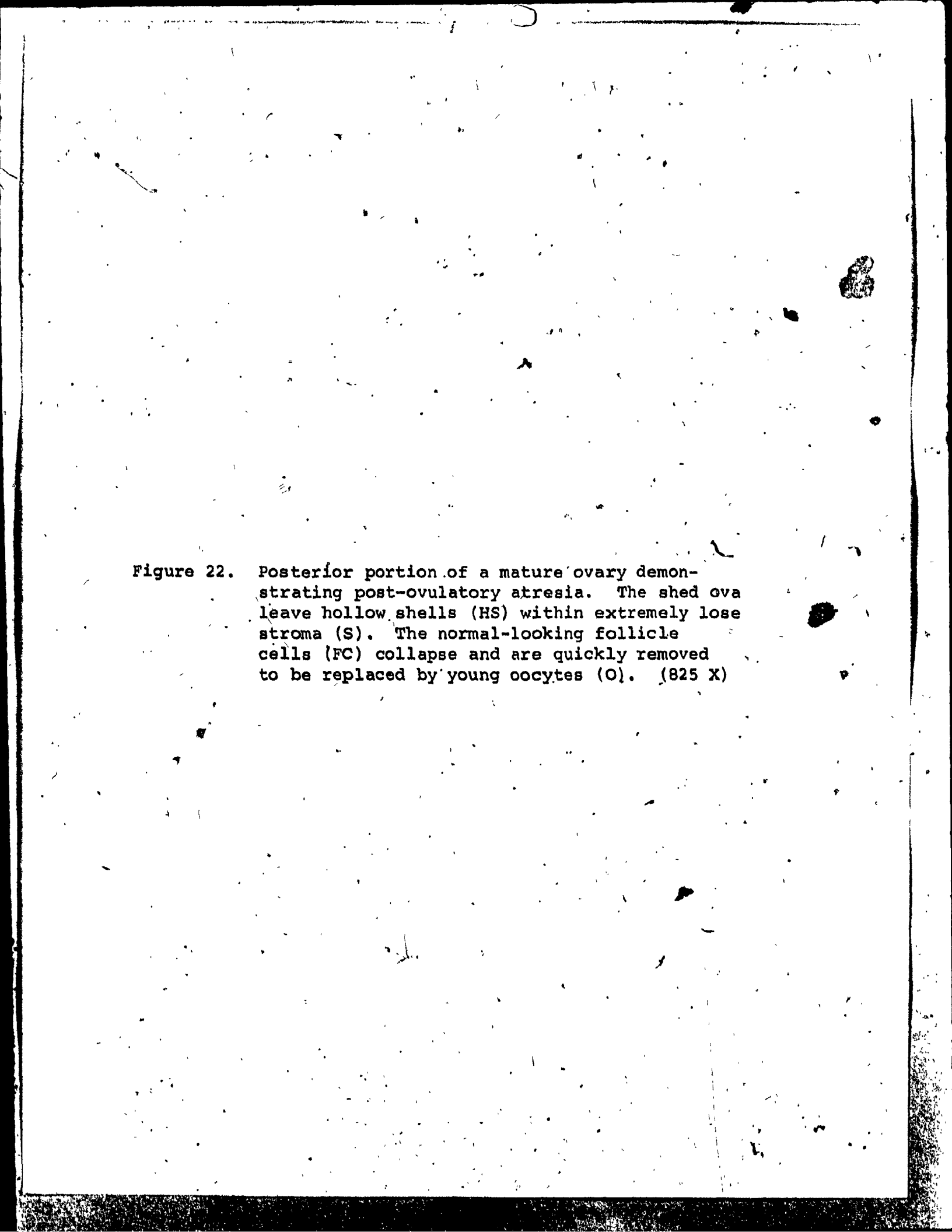


Figure 22. Posterior portion of a mature ovary demonstrating post-ovulatory atresia. The shed ova leave hollow shells (HS) within extremely lose stroma (S). The normal-looking follicle cells (FC) collapse and are quickly removed to be replaced by young oocytes (O). (825 X)

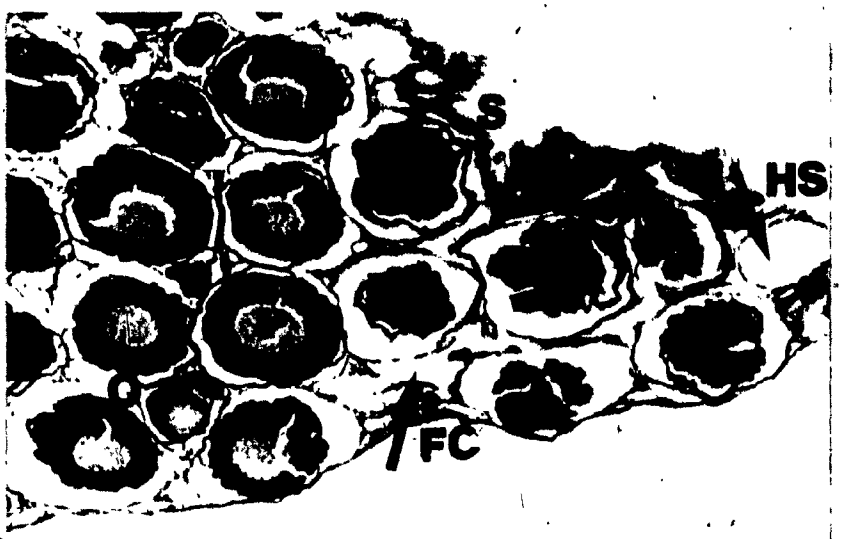

S
HS
FC

Figure 23. Earliest form of oocyte damage associated mainly with 0.01 mg/l HCN. Note the retreating acidophilia (A) and loss of yolk vesicles in the ooplasm (O) at the right. The nuceoli (Nu) are displaced from a shrinking nuclear membrane. (825 X)

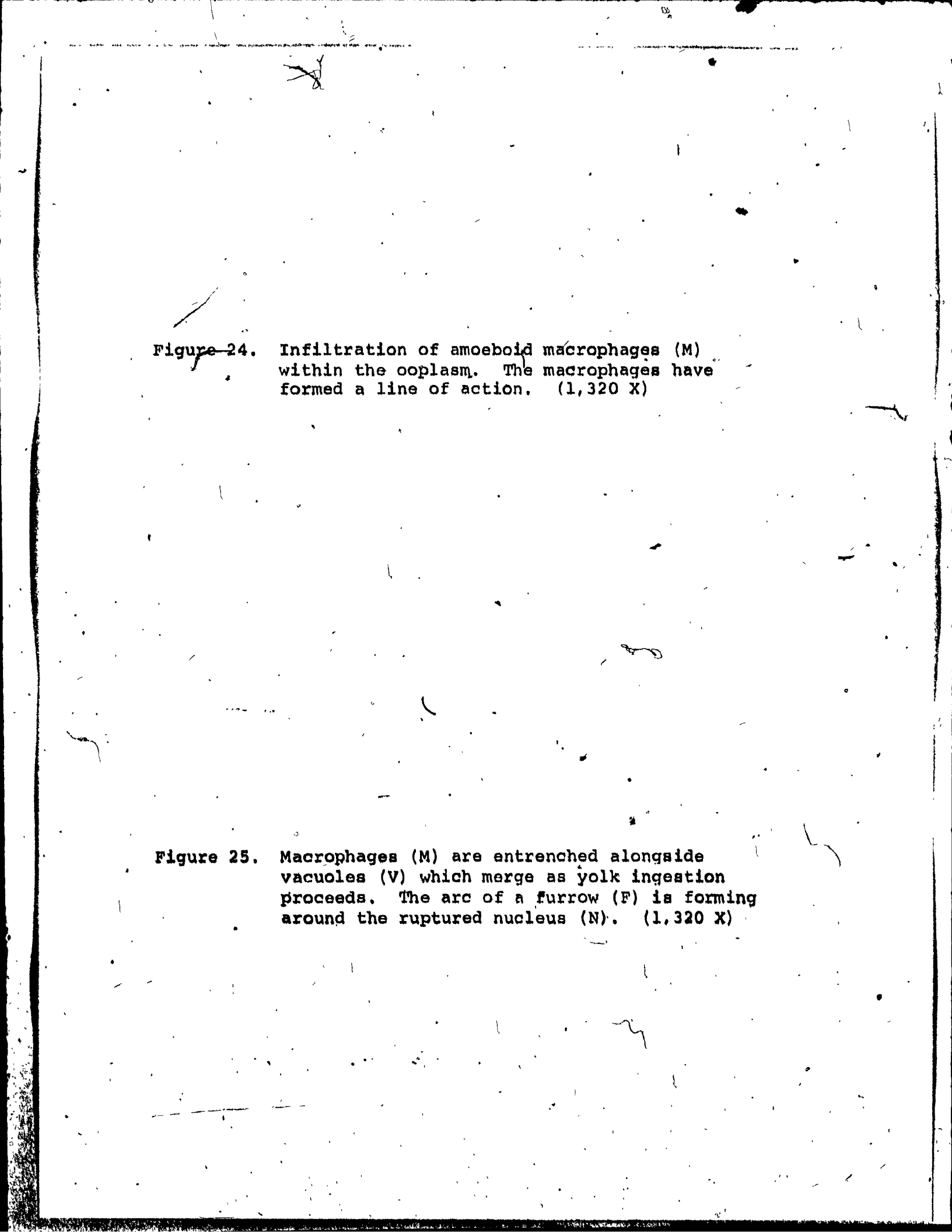


Figure 24. Infiltration of amoeboid macrophages (M) within the ooplasm. The macrophages have formed a line of action. (1,320 X)

Figure 25. Macrophages (M) are entrenched alongside vacuoles (V) which merge as yolk ingestion proceeds. The arc of a furrow (F) is forming around the ruptured nucleus (N). (1,320 X)

Figure 26. Further degeneration resulting in the formation of clear furrows (F) of collapsed cooplasm. (825 X)

Figure 27. Severe erosion of the ooplasm continues until concentric swirls of furrows (F) are formed. Each furrow is still lined by macrophages (M). The covering membranes become separated (S) from the egg surface. Phagocytes (P) are occasionally encountered especially at the periphery. (825 X).

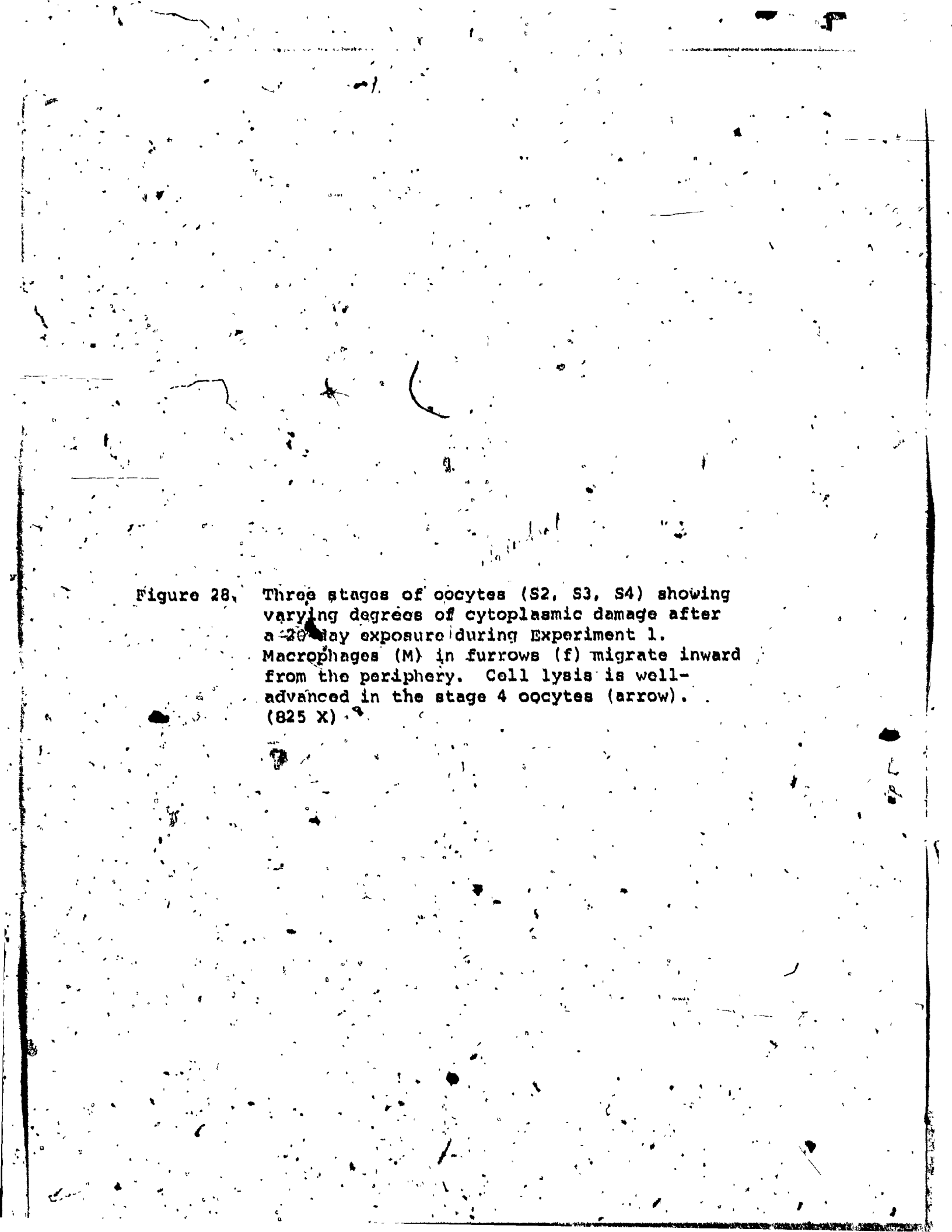
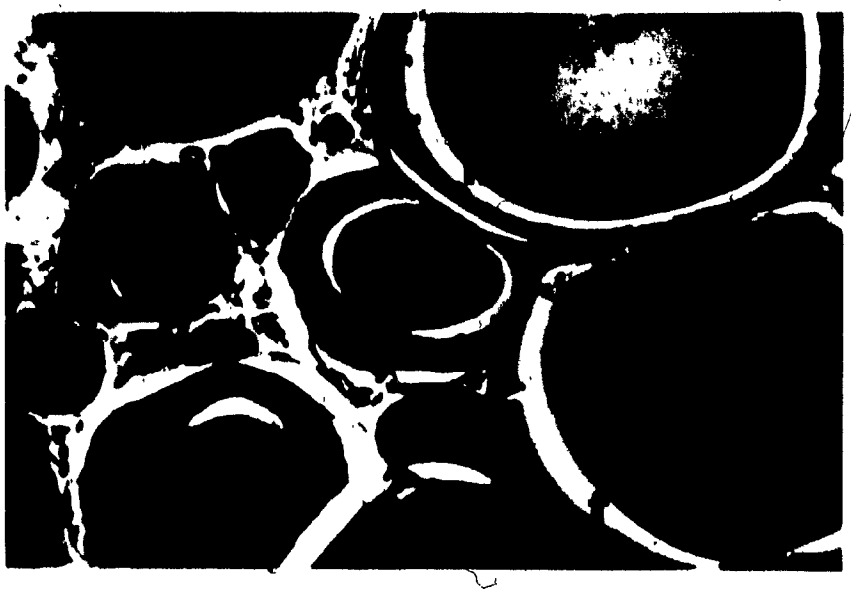


Figure 28. Three stages of oocytes (S2, S3, S4) showing varying degrees of cytoplasmic damage after a 20 day exposure during Experiment 1. Macrophages (M) in furrows (f) migrate inward from the periphery. Cell lysis is well-advanced in the stage 4 oocytes (arrow). (825 X)



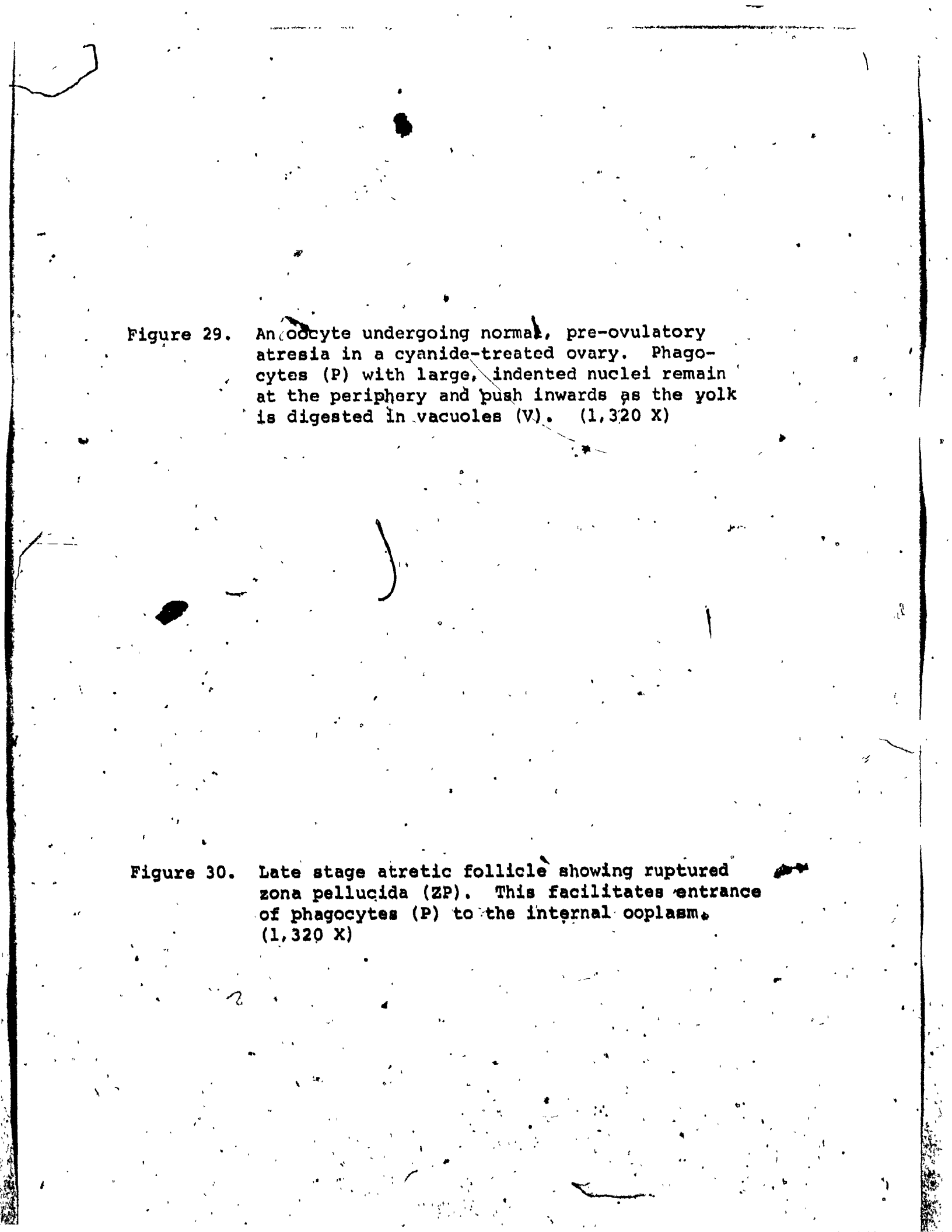


Figure 29. An oocyte undergoing normal, pre-ovulatory atresia in a cyanide-treated ovary. Phagocytes (P) with large, indented nuclei remain at the periphery and push inwards as the yolk is digested in vacuoles (V). (1,320 X)

Figure 30. Late stage atretic follicle showing ruptured zona pellucida (ZP). This facilitates entrance of phagocytes (P) to the internal ooplasm. (1,320 X)


ZP
P

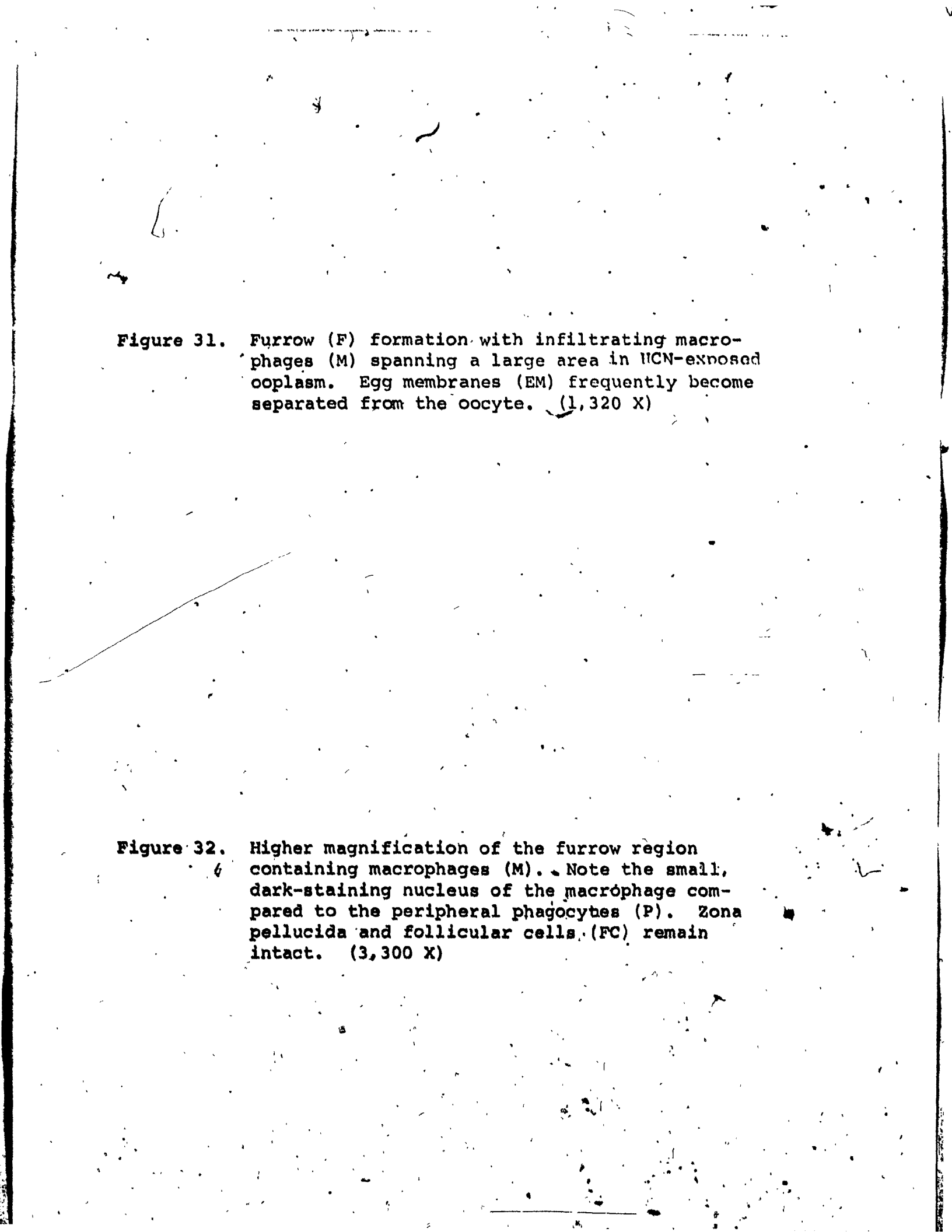


Figure 31. Furrow (F) formation with infiltrating macrophages (M) spanning a large area in HCN-exposed ooplasm. Egg membranes (EM) frequently become separated from the oocyte. (1,320 X)

Figure 32. Higher magnification of the furrow region containing macrophages (M). Note the small, dark-staining nucleus of the macrophage compared to the peripheral phagocytes (P). Zona pellucida and follicular cells (FC) remain intact. (3,300 X)


F


M

Figure 33. Early modification of the ellipsoidal contour of oocytes previously exposed to cyanide. (825 X)

Figure 34. Extreme ellipsoid in a stage 5 oocyte after 20 days exposure to 0.02 mg/l HCN in experiment 1. (1,320 X)

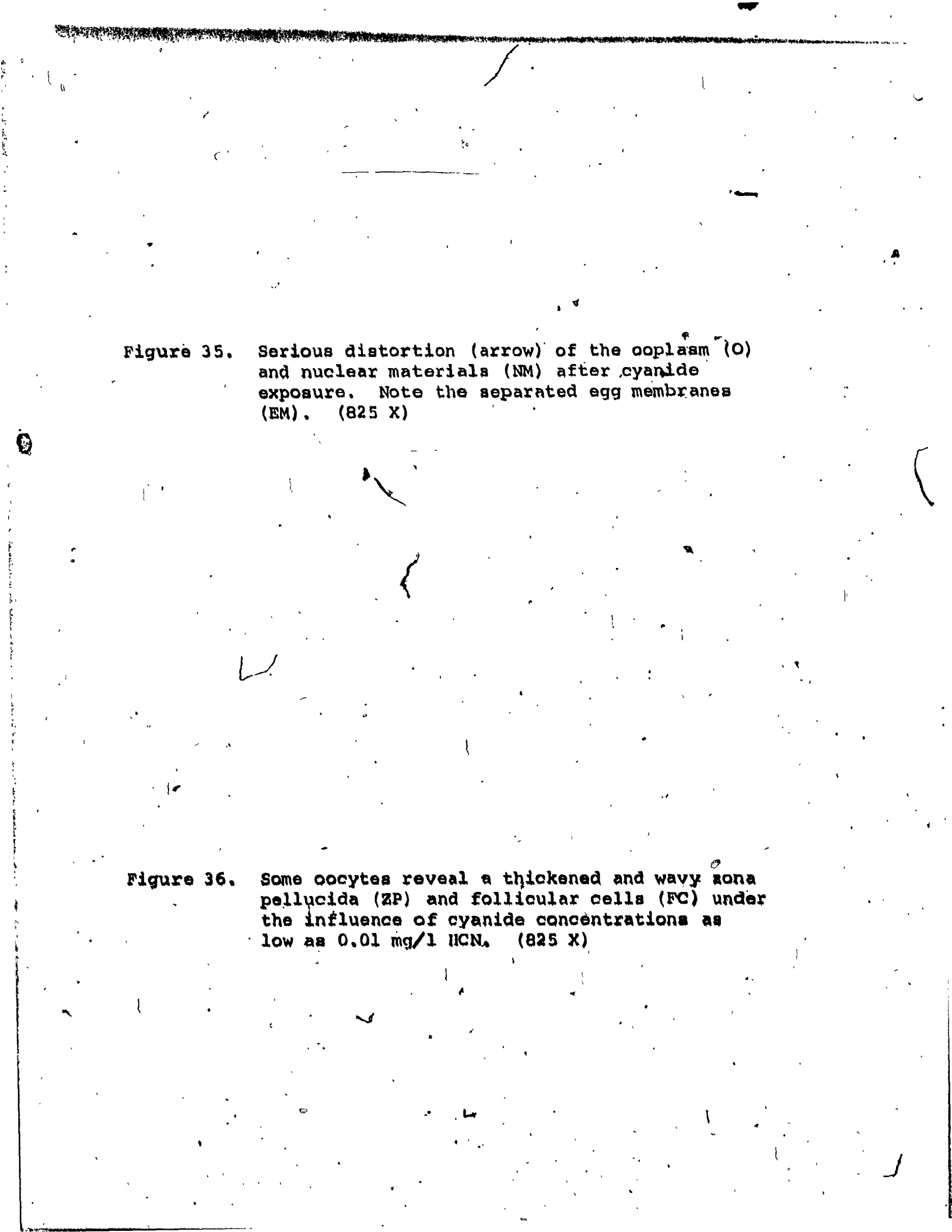


Figure 35. Serious distortion (arrow) of the ooplasm (O) and nuclear materials (NM) after cyanide exposure. Note the separated egg membranes (EM). (825 X)

Figure 36. Some oocytes reveal a thickened and wavy zona pellucida (ZP) and follicular cells (FC) under the influence of cyanide concentrations as low as 0.01 mg/l HCN. (825 X)

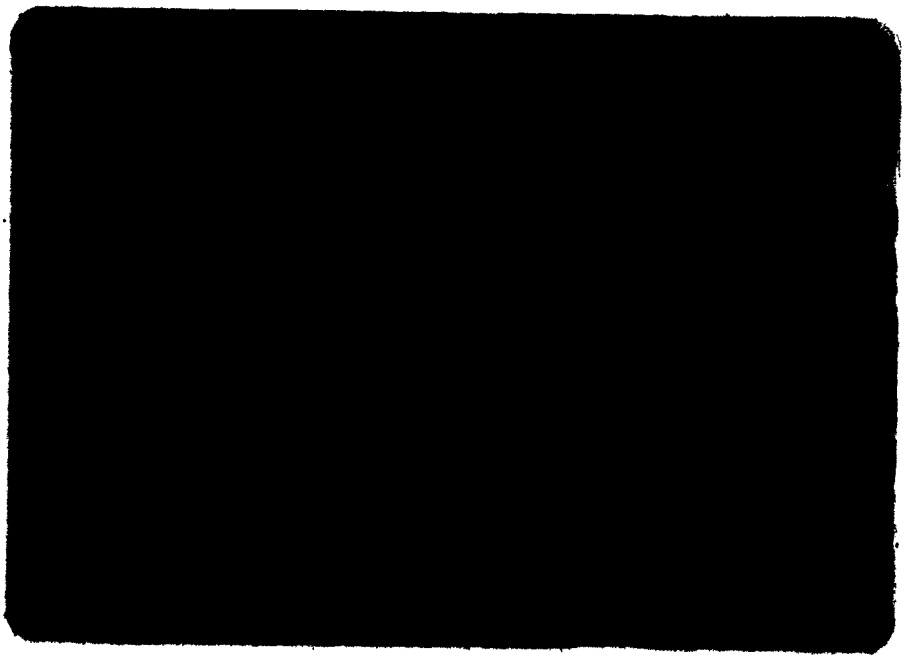
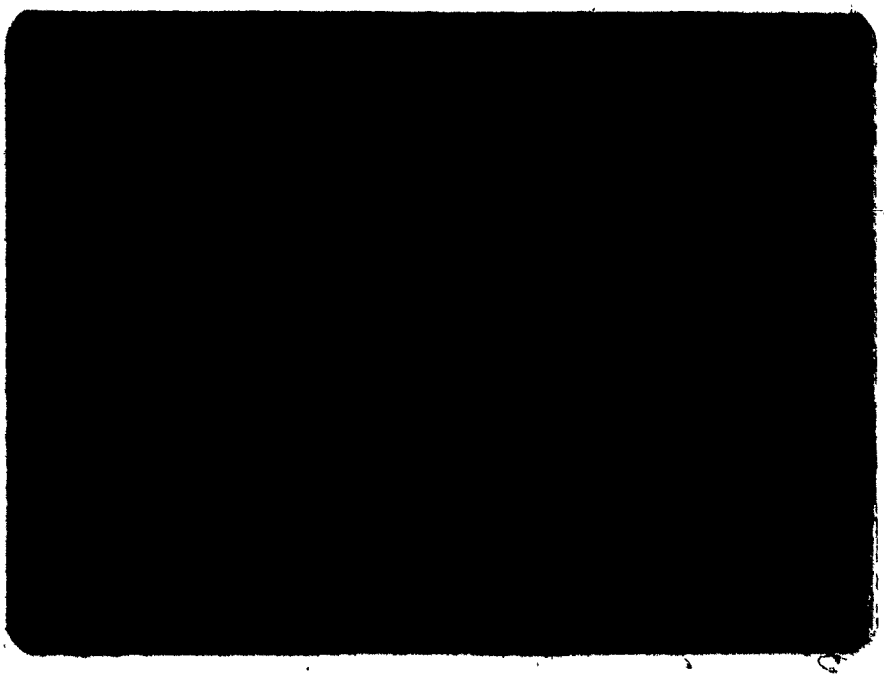


Figure 37. Control stage 5 oocyte during Experiment 1. The cytoplasm displays PAS-positive granules radiating from the perinuclear area. A single band of 2° yolk is present at the periphery and another immediately outside the nucleus. (825 X)

Figure 38. Oocyte of comparable size in cyanide-treated ovary of the same experiment. Note the patchwork effect of Balbiani (B) fragments indicating a lag in development. (825 X)



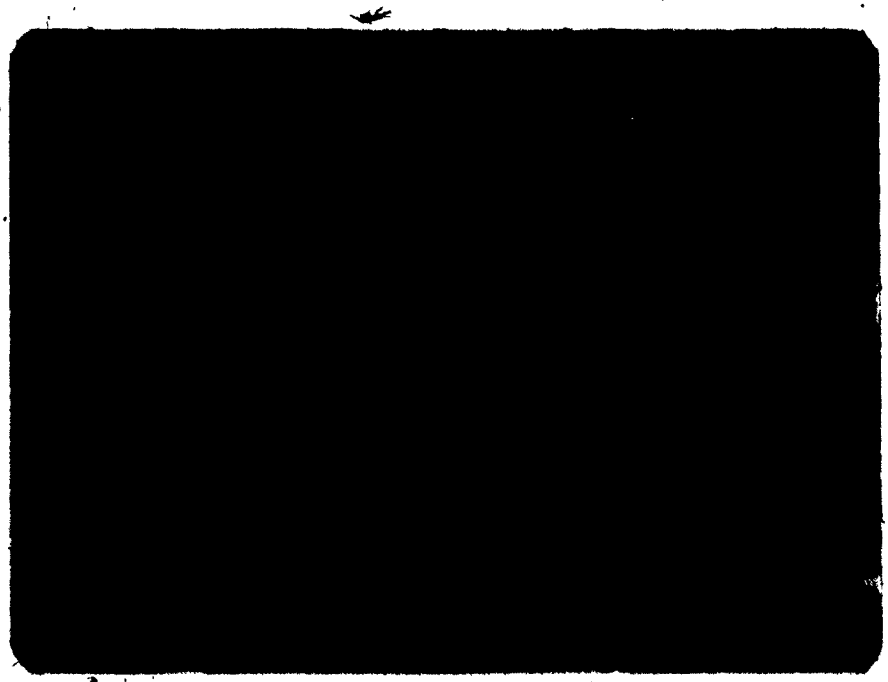
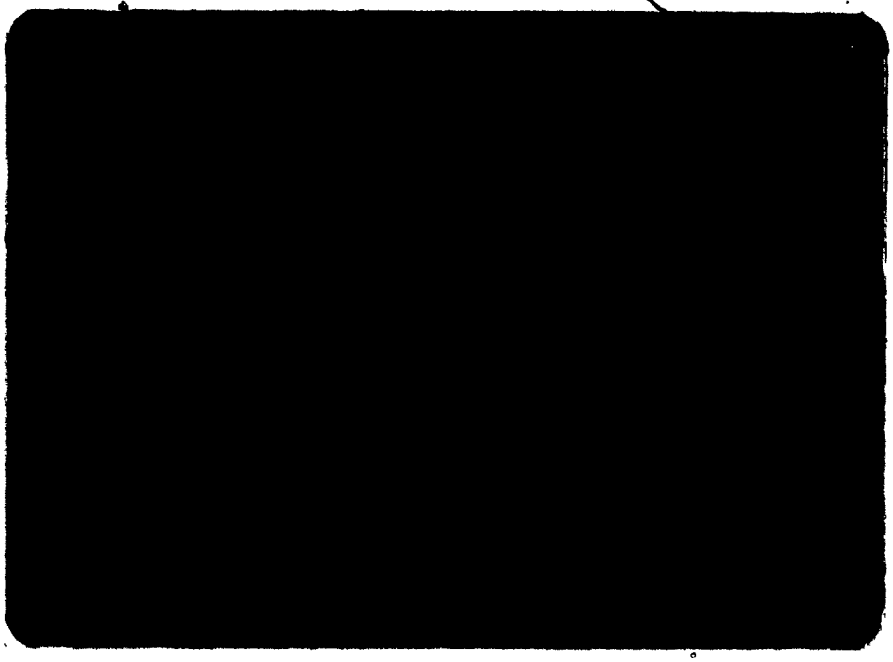


Figure 39. Control late stage 5 oocyte during Experiment 2, displaying a peak period of secondary yolk development (arrows). The nucleus (N) is irregular in outline and lacking lampbrush chromosomes. (825 X)

Figure 40. Large accumulations of granules (Gr) brilliantly stained with PAS may occur as early as stage 3. (1,320 X)



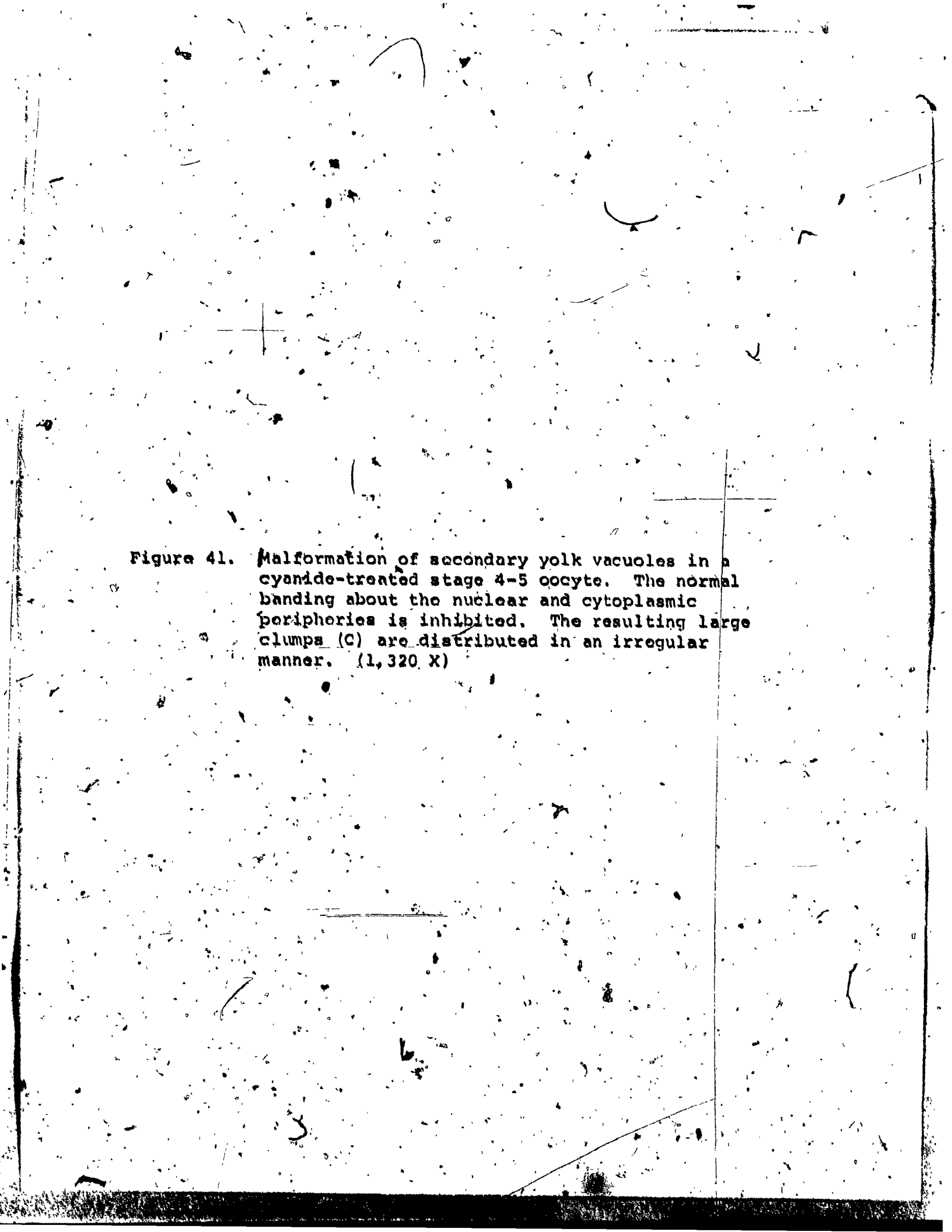


Figure 41. Malformation of secondary yolk vacuoles in a cyanide-treated stage 4-5 oocyte. The normal banding about the nuclear and cytoplasmic peripheries is inhibited. The resulting large clumps (C) are distributed in an irregular manner. (1,320 X)

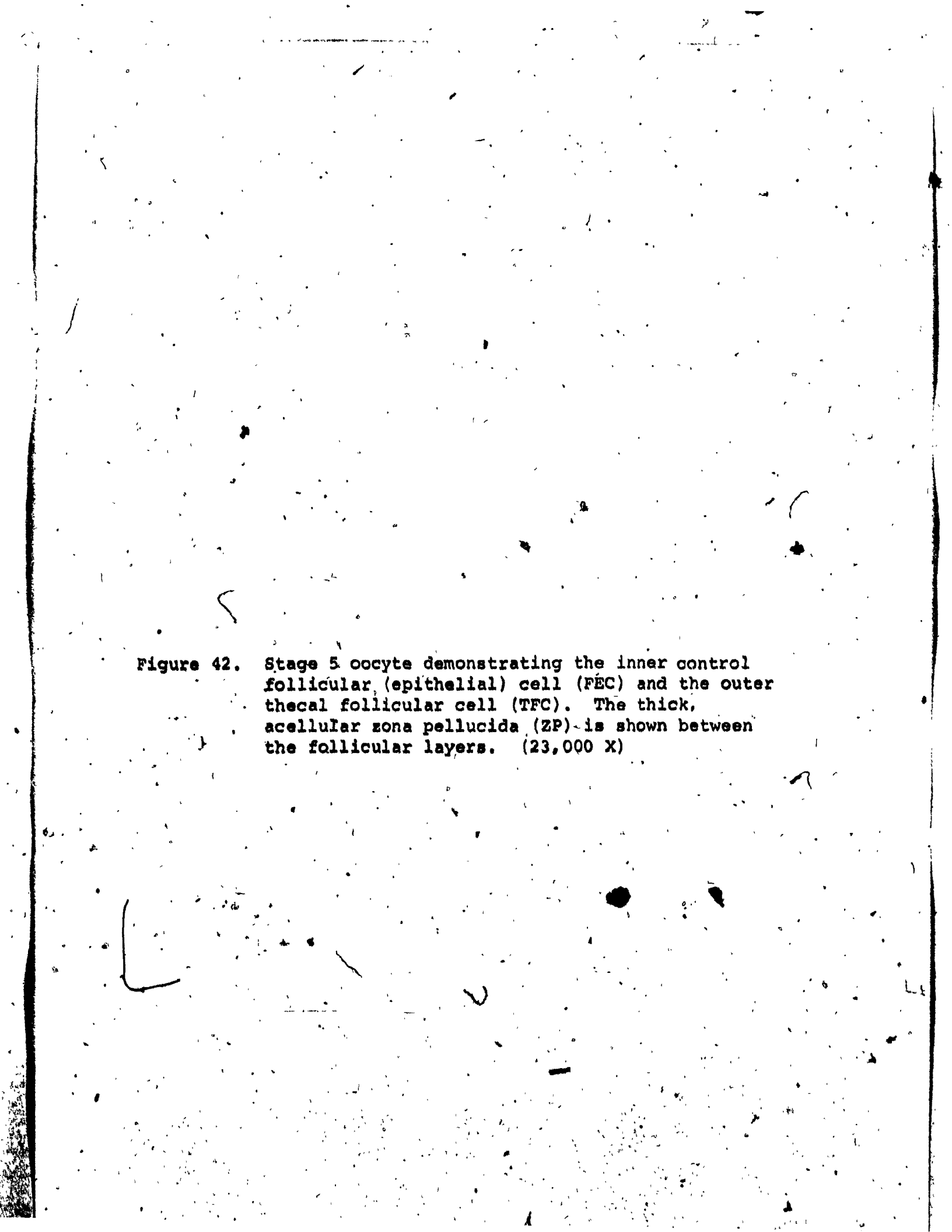


Figure 42. Stage 5 oocyte demonstrating the inner control follicular (epithelial) cell (FEC) and the outer thecal follicular cell (TFC). The thick, acellular zona pellucida (ZP) is shown between the follicular layers. (23,000 X)

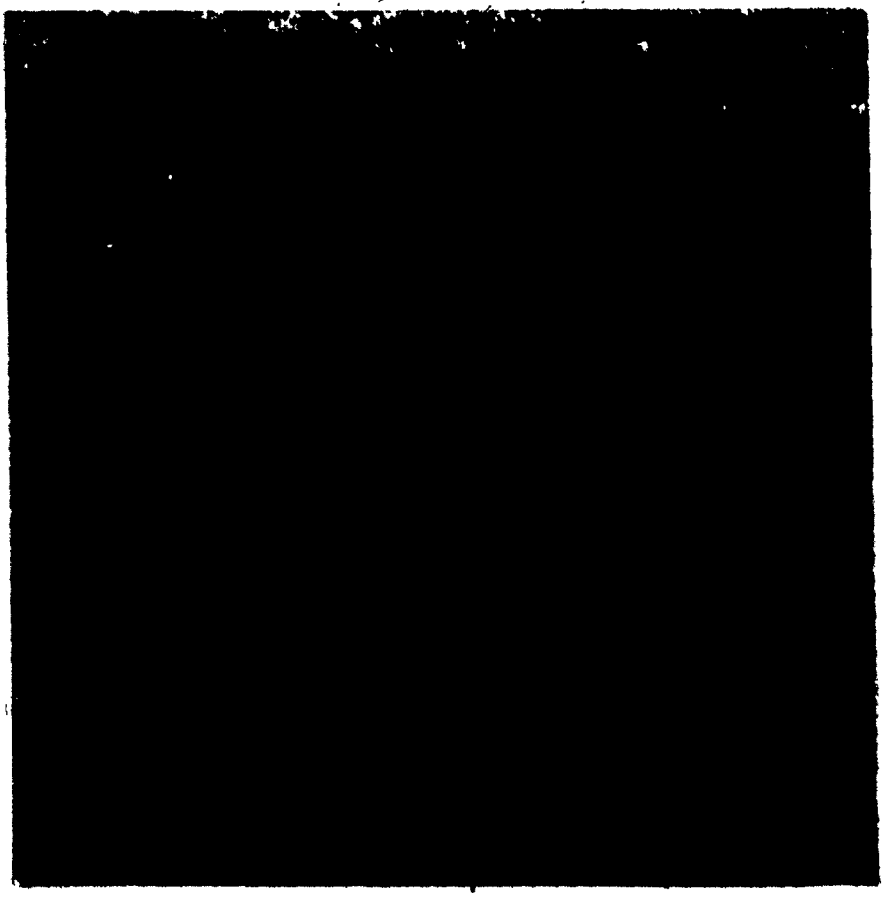


Figure 43. Various shapes of mitochondria (M) are present in a control, late-stage oocyte. The follicular cell mitochondria (FCM) is round in form whereas those within the central ooplasm vary from oval to long and coiled (arrows). (37,000 X)

Figure 44. A mitochondrion (M) from the central cytoplasm of a late stage oocyte within a control ovary. The double membrane is irregular on the outer surface. The inner membrane displays transverse cristae (C). The tubular cristae are embedded in a finely granular matrix containing several dense granules (DG). (193,000 X)

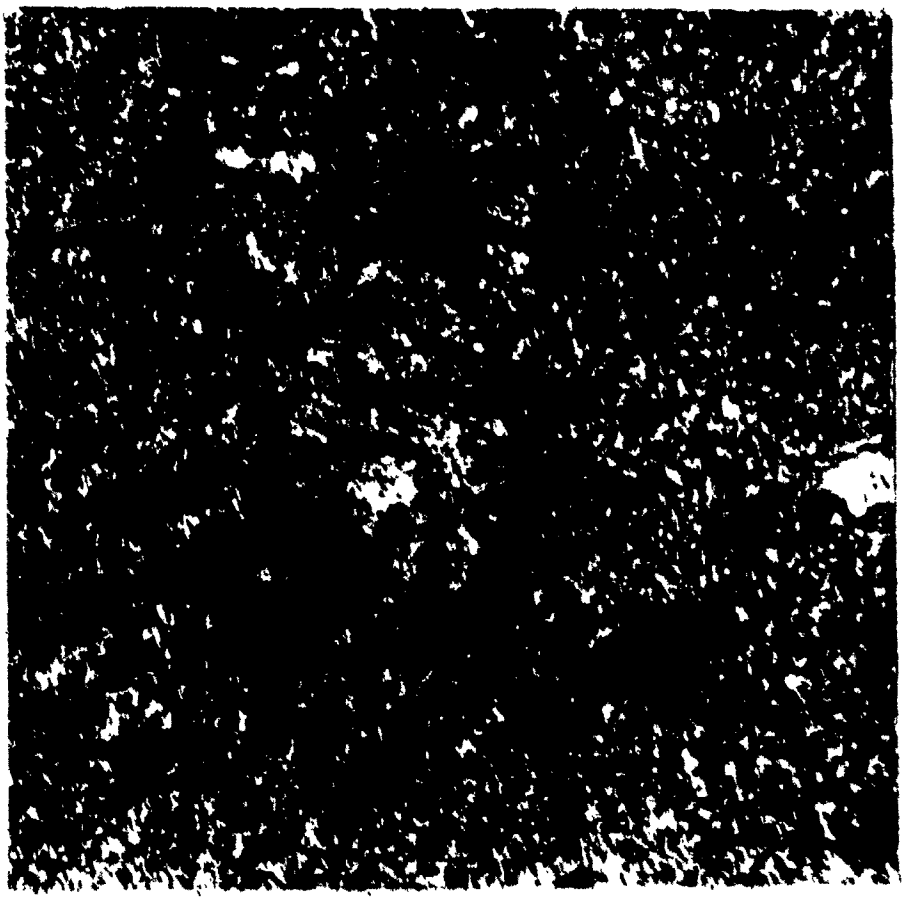


Figure 45. Control follicular cell illustrating a mitochondrion (M) which is bounded by a smooth-contoured outer membrane. Parallel cristae (C) are present in the ground matrix. Small, dense matrix granules (G) are also evident. (124,000 X)

Figure 46. A follicular cell mitochondria (M) after 20 days exposure to 0.01 mg/l HCN. The double membranes appear laminated (L) and the ruptured area (arrows) results in a release of the internal contents. The matrix contains many granules (G). (92,000 X)

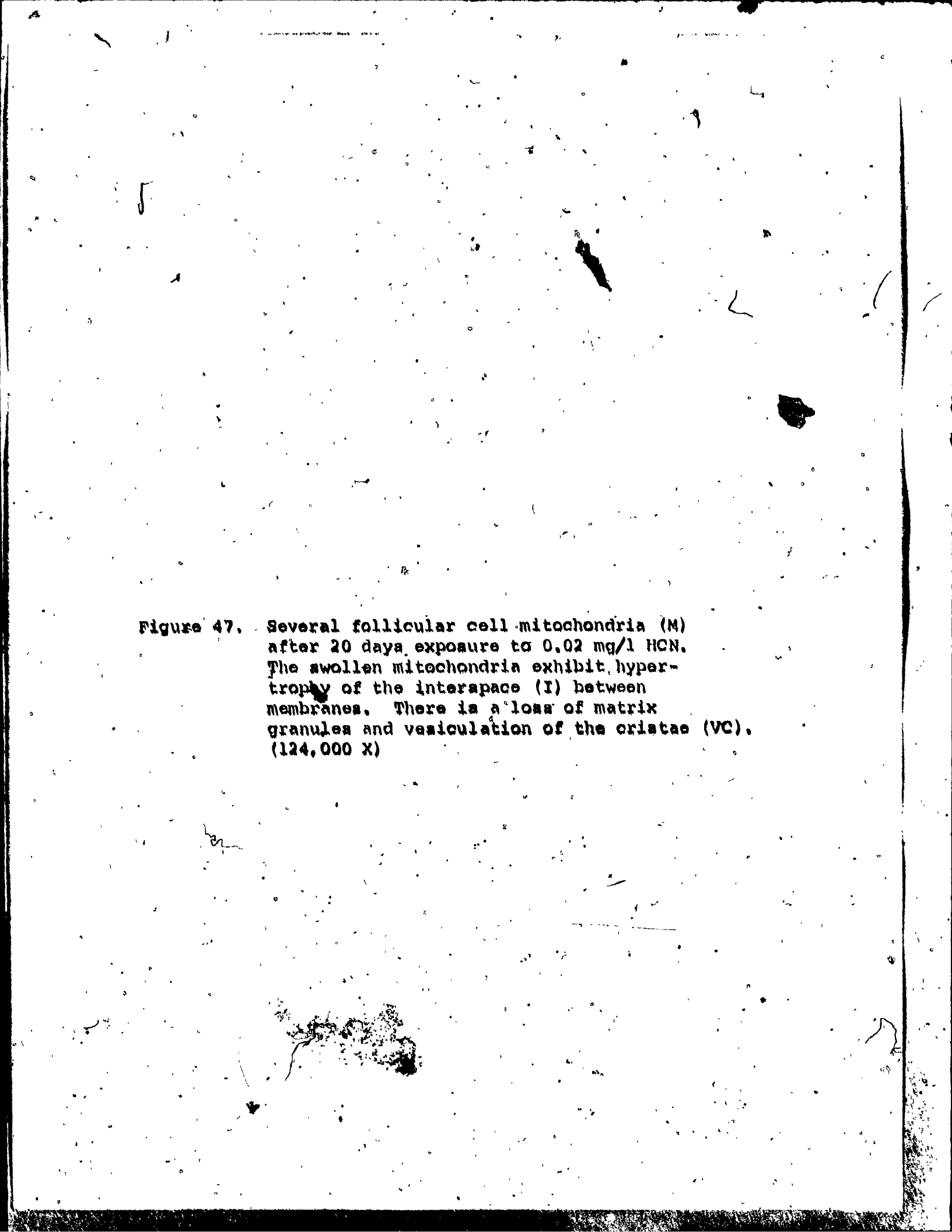


Figure 47. Several follicular cell mitochondria (M) after 20 days exposure to 0.02 mg/l HCN. The swollen mitochondria exhibit hypertrophy of the interspace (I) between membranes. There is a loss of matrix granules and vesiculation of the cristae (VC). (124,000 X)

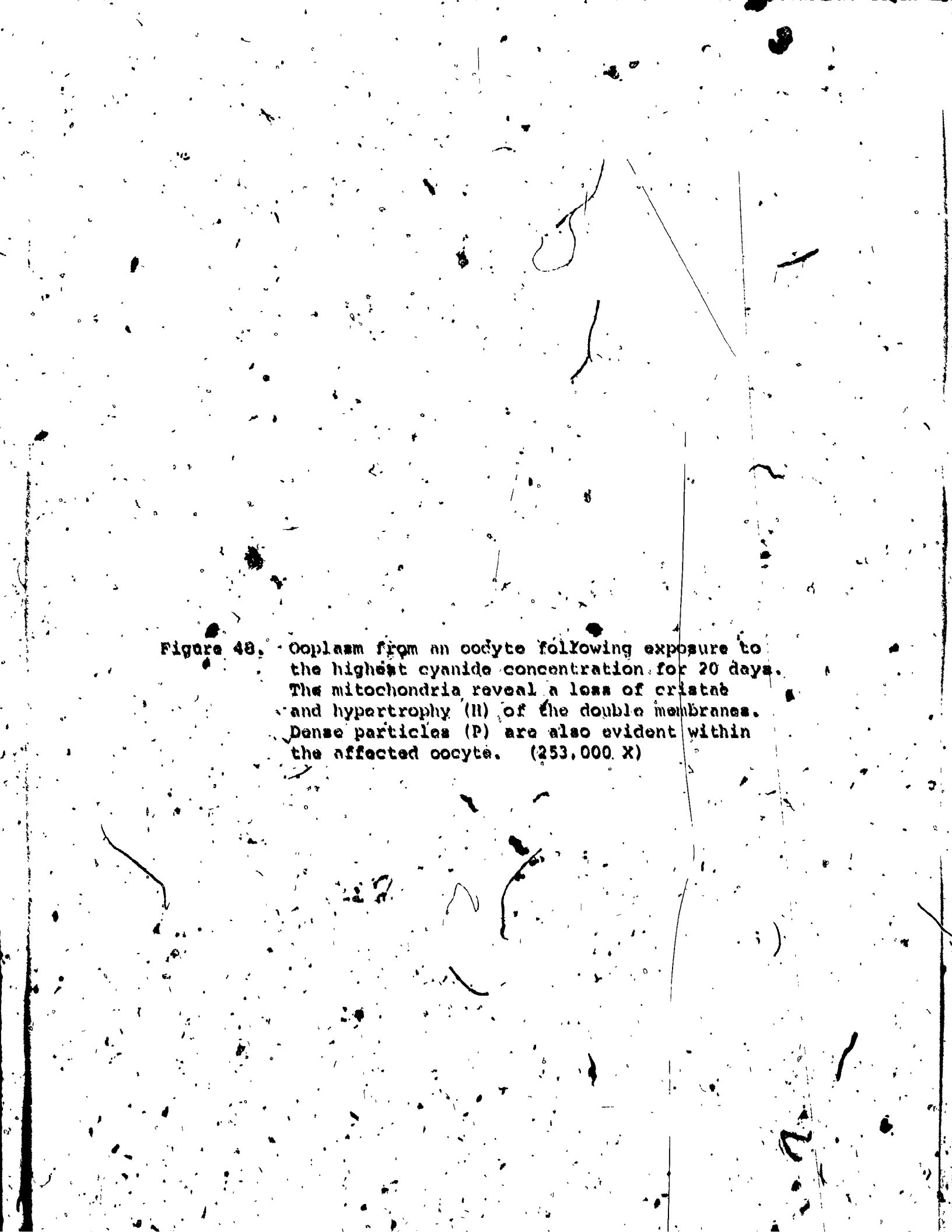


Figure 48. Ooplasm from an oocyte following exposure to the highest cyanide concentration for 20 days. The mitochondria reveal a loss of cristae and hypertrophy (H) of the double membranes. Dense particles (P) are also evident within the affected oocyte. (253,000 X)

Appendix A

Alternative terminology for Balbiani body:

Yolk nuclear complex

Yolk nucleus

Dotterkern

Balbianis vitelluis body

Corp vitellin

Cytocenter

Chromidia

Synthetic center

Refer to: Beams and Kessels (1973)