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SOME ULTRASTRUCTURAL, BIOCHEMICAL AND PHYSIOLOGICAL CHANGES INDUCED BY THE CARCINOGEN 4-NITROQUINOLINE N-OXIDE IN THE FLAGELLATE CRITHIDIA FASCICULATA.

by

LEONARD A. COHEN

A dissertation submitted to the Graduate Faculty in Biology in partial fulfilment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1972

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

June 30, 1972
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TABLE OF CONTENTS

LIST OF TABLES, CHARTS, DIAGRAMS AND FIGURES

Tables

Charts

Diagrams

Figures

ABBREVIATIONS

Act-D: actinomycin D; AF: acriflavine; AMP: adenosine monophosphate; 4-AQO: 4-aminoquinoline N-oxide; AT: 2,4 aminotriazole; DAB: 3,3'-diaminobenzidine tetrahydrochloride; DNP: 2,4-dinitrophenol; GPO: X-glycerophosphate oxidase; HAQO: hydroxylaminoquinoline N-oxide; IC_{50}: 50% inhibitory concentration; kDNA: kinetoplast DNA; LC_{50}: 50% lethal concentration; mDNA: mitochondrial DNA; NAD: nicotinamide adenine dinucleotide; nμDNA: nucleolar DNA; 4-NQO: 4-nitroquinoline N-oxide; PCV: packed cell volume; RD: respiratory deficient; RNP: ribonucleoprotein particle; rRNA: ribosomal RNA: RSM: ribosomal suspension medium; TCA: trichbroacetic acid; TEA: triethanolamine; TTC: triphenyltetrazolium chloride; XP: Xeroderma pigmentosum.

Only the hand that erases

can know the true thing.

Meister Eckhart

INTRODUCTION

Study of any chemical carcinogen ordinarily progresses through three phases: 1) discovery of carcinogenicity; 2) synthesis of homologs and analogs in order to correlate chemical structure with carcinogenicity; 3) charting interactions with cellular macromolecules, and effects of the carcinogen on macromolecular syntheses with the ultimate goal being the detection and selection of the precise interactions that engender the carcinogenic process. A similar sequence is discernable in development of knowledge of the carcinogen 4-nitroquinoline N-oxide (4-NQO) (see diagram 1), and suggested the work described here.

Carcinogenicity of this water-soluble, relatively stable yet highly toxic carcinogen was discovered by Nakahara et al. (1957) by skin painting of mice. Soon thereafter its potency was demonstrated in a wide variety of tissues and organisms with the conspicuous exception of rat liver; for reviews see various chapters in the monograph edited by Endo, Ono, and Sugimura (1970). The history of the synthesis of 4-NQO and discovery of its antimicrobial activity

and carcinogenicity was reviewed by Ochiai (1967).

The second phase in development of knowledge of 4-NQO centered on synthesis of congeners of 4-NQO to ascertain which functional groups were critical determinants of carcinogenicity. The 4-nitro and the N-oxide groups proved essential for carcinogenicity (Nakahara et al., 1957). Activity was retained and in some instances enhanced by alkyl or halide substituents on the ring carbons (Nakahara et al., 1958, Takayama, 1962); the 6,7-dimethyl derivative proved exceptionally carcinogenic (Lacassagne et al., 1966). Also, a close parallel in potency was shown between derivatives which could undergo non-enzymatic substitution at the 4-nitro group by SH compounds (as described in diagram 2) and carcinogenicity (Fukui et al., 1959; Hayashi, 1959; Nakahara & Fukuoka, 1959; Okamoto & Itoh, 1963).

The third phase was ushered in by demonstration by several physical-chemical techniques that 4-NQO and 4-HAQO interacted with DNA *in vitro* (Malkin & Zahalsky, 1966; Nagata et al., 1966b; Okano & Uekama, 1967) and *in vivo* (Ikegami et al., 1969/1970; Matsushima et al., 1967; Tada et al., 1967; Tada et al., 1970; Tada & Tada, 1971).

LEGENDS DIAGRAM 1 & 2

Diagram 1: canonical formulae and ring numbering system for 4-NQO.

Diagram 2: sulfhydryl substitution reaction, R = cysteine, methionine, glutathione.

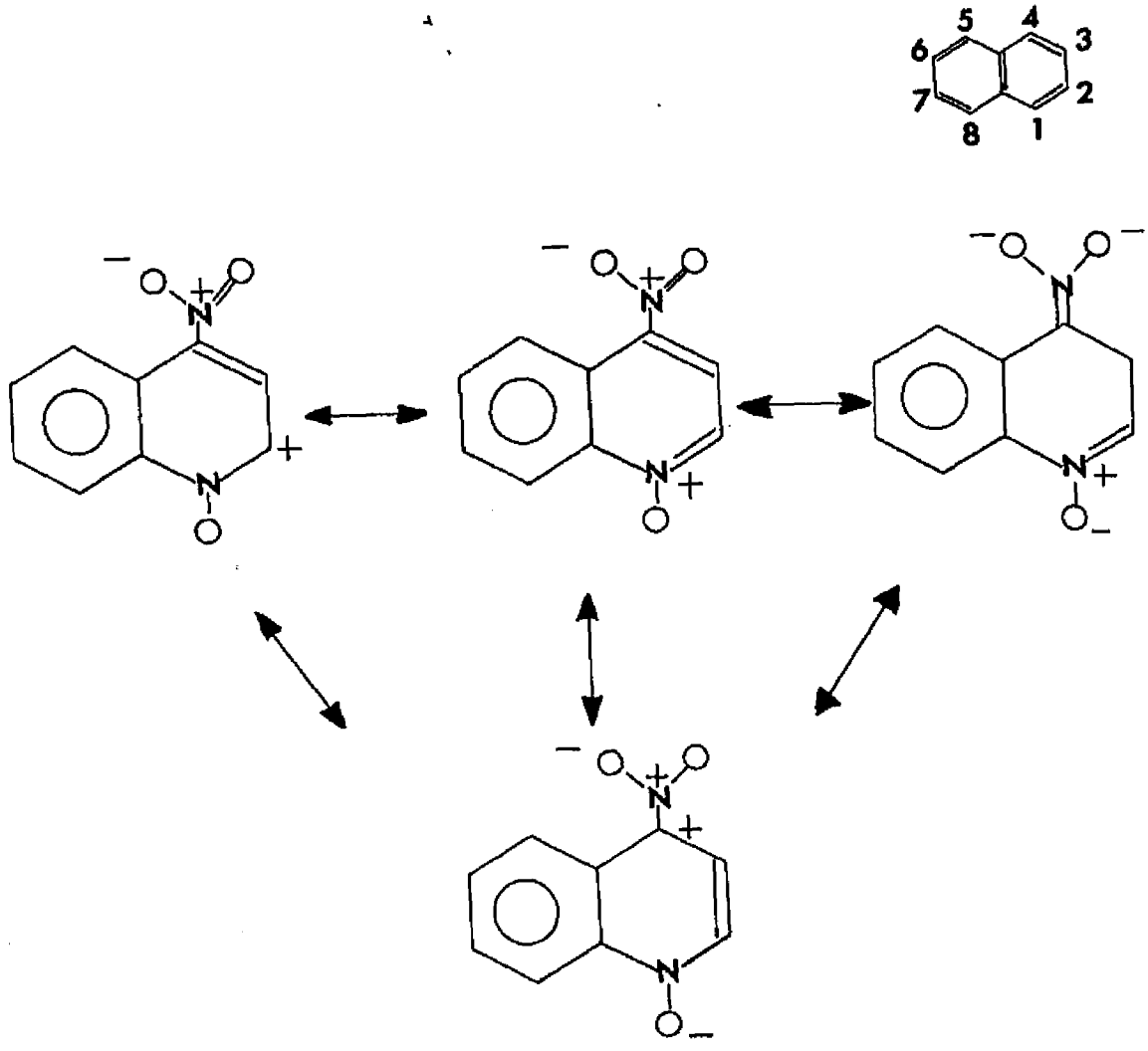


DIAGRAM 1

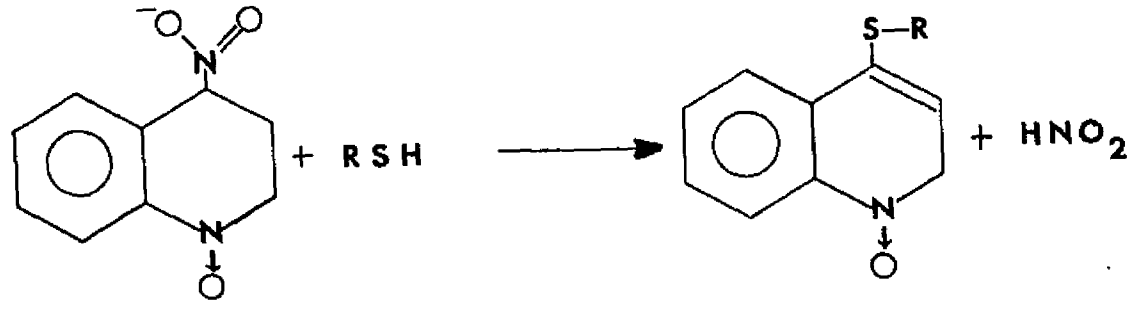


DIAGRAM 2

In addition, interaction of the highly carcinogenic derivative of 4-NQO, 4-HAQO, with DNA shown indirectly by genetic methods in bacteriophage (Ishizawa & Endo, 1967). 4-NQO also induced mutation but failed to block infectivity in tobacco mosaic virus, suggesting specific interaction with viral RNA (Endo et al., 1961). Intracellularly, 4-NQO is enzymatically altered before binding to DNA: the NO_2 group is reduced by nitro reductases, first to HAQO and then to the non-carcinogenic AQO in fungi (Okabayashi & Yoshimoto, 1962), bacteria (Fukuda & Yoshimoto, 1972; Kato et al., 1970), and liver (Sugimura et al., 1966). The unusually rapid and intense carcinogenicity of the reduction product, 4-HAQO, was demonstrated by Endo & Kume (1963, 1965); Kawazoe et al. (1967); Shirasu (1965). Moreover, a close correlation was found between carcinogenicity of 4-NQO derivatives and susceptibility to enzymatic reduction to the hydroxy compound (Araki et al., 1970), prompting the idea that 4-HAQO was the proximate, i.e., ultimate, carcinogen and 4-NQO a pre-carcinogen (Miller, 1970). The likelihood that 4-NQO is converted to a highly reactive nitroso (free radical) reaction product of 4-HAQO before binding to DNA has been discussed by

Nagata et al. (1966a) and Nobuku et al. (1968).

Investigations of 4-NQO action are now in a fourth phase of development with interest centering on intracellular regulation. The major findings have been that (a) 4-NQO blocks the enzymatic synthesis of RNA (Paul et al., 1966, 1967, 1969) in isolated calf thymus nuclei and Hela cells; and (b) incorporation of labeled carcinogen and inhibition of DNA synthesis and eventually RNA and protein synthesis were directly correlated (Kuroki et al., 1969, 1970).

The first two phases outlined have been amply studied but important questions remain unresolved for phases three and four; the specific issues dealt with here are:

1) Although it is established that 4-NQO interacts with cellular DNA, no studies distinguish between mitochondrial and nuclear DNA -- an important distinction in view of the celebrated Warburg hypothesis that impaired respiration generally accompanies carcinogenesis and indeed may be its root cause (Racker, 1972); Gause's studies on respiratory-deficient bacterial mutants (1966) and Wunderlich's (1971, 1972) recent reports that certain carcinogenic hydrocarbons selectively

interact with mitochondrial DNA -- point up the question: does 4-NQO interact preferentially with cellular DNA's and if so which DNA -- mDNA or nDNA?; and,

2) which are the earliest detectible changes after the initial interaction notably in morphology and macromolecular synthesis?

3) which biochemically and morphologically invariant hereditary changes are induced by 4-NQO?

Questions 1 and 2 are dealt with in section 1 and question 3 in section 2.

We sought to answer these questions by means of a protozoan which permits a unique morphological distinction between nDNA and mDNA, and by correlating biochemical, ultrastructural, and cytochemical changes. The following paragraphs describe Crithidia fasciculata and its advantages and disadvantages as a model system; methodology will then be discussed.

Crithidia is a nonpathogenic insect gut parasite easily grown axenically in crude or defined media supplemented with heme (usually supplied as hemin); it cannot synthesize protoporphyrin (Lwoff, 1951), and

available heme is almost entirely lacking in such materials as bacteriological peptones and yeast extracts. Moreover, Crithidia is inexpensively grown in bulk for biochemical analysis. Crithidia has a tough outer cell wall, so enabling the preparative techniques of electron microscopy and general cytochemistry to be applied with minimal difficulty. Also, it is clonable on solid substrates (agar) thereby assuring a homogeneous population for biochemical studies and selection of mutants when desirable (Cohen & Zahalsky, 1968).

Crithidia has been well characterized morphologically (Brooker, 1971; Kusel et al., 1967) and biochemically. Despite the fact that it is an aerobic fermenter, i.e., it incompletely oxidizes sugars (von Brand, 1966), Hunter (1957) has shown that Crithidia can follow the conventional tricarboxylic acid pathway aerobically, and that the degradation of glucose is a typical glycolysis. The respiratory chain located in the long, single mitochondrion (Margulies, 1970; Vickerman, 1962) consists of cytochromes $\underline{a}$-$\underline{a}_3$, $\underline{b}$-$\underline{c}_{555}$ coenzymes $\underline{Q}_9$ and the unusual terminal oxidase, cytochrome oxidase $\underline{o}$ (Baernstein, 1963; Hill et al., 1968; Hill & Anderson, 1970; Hill & White, 1968).

Other features argue for its use as a model system with which to pursue the questions just posed. Aside from being eukaryotic, the system is accessible to a wide variety of experimental procedures ranging from electron microscopy to cell fractionation techniques; moreover, it is a reproducible system with minimal variability, in contrast to metazoan systems where age, sex, route of administration, hormonal status, tissue and organ specificities, and immunity phenomena complicate interpretation of experiments. See Frenkel, (1969); and Huseby, (1969) for a detailed discussion of models and their uses and misuses, in the study of oncology.

A serious disadvantage of Crithidia is the fact that this organism is not known to have any sexuality, (i.e., meiosis and fertilization) nor is it known to harbor phages that could be employed for genetic transfers. This precludes genetic analysis to characterize mutant strains and prevents ready decision as to whether a mutation is cytoplasmic or nuclear.

Two cytological features of Crithidia recommend it for study of DNA-binding agents, such as 4-NQO. In the long single mitochondrion, near the basal body of the flagellum is a structure unique to the Order

Kinetoplastidae (Honigberg, 1967) called the kinetoplast. The kinetoplast is a specialized section of the mitochondrion situated in the inner matrix containing up to 30% of the total cellular DNA (Riou & Pautrizel, 1969; Steinert et al., 1969). It has long been known that the kinetoplast is susceptible to damage (Simpson & DaSilva, 1971) by several drugs which bind to DNA such as acriflavine (Cosgrove, 1966; Guttman & Eisenman, 1965; Hill & Anderson, 1969; Kusel et al., 1967; Simpson, 1968; Stuart & Hanson, 1967; Trager & Rudzinska, 1964) and ethidium bromide (Steinert et al., 1969; Thirion & Kupersztein, 1968). Exposure to these drugs for 3-4 days induces a condition, known as dyskinetoplasty, in which kinetoplast ultrastructure collapses leaving an abortive mitochondrion and a condensed electron opaque mass of what had been DNA fibrils, at concentrations which do not affect the nucleus. The simultaneous dissappearance of kDNA bands in cesium chloride gradients indicate that dyskinetoplasty is the result of preferential damage to kDNA (Renger & Wolstenholme, 1971; Simpson, 1968; Steinert & Van Assel, 1967). Hence induction of dyskinetoplasty can be taken as an index of a selective interaction with kDNA.

A second useful cytological feature of Crithidia is the presence in the nucleus of a single prominant nucleolus possessing ultrastructural features characteristic of a metazoan cell nucleolus. The nucleolus, like the kinetoplast, is susceptible to alteration by agents which bind selectively to DNA, in this case, however, by a different class of compounds, typified by actinomycin D (Busch & Smetana, 1970; Simard, 1970). Exposure to actinomycin results in nucleolar segregation in mammalian cells, (Reynolds et al., 1964; Schoefl, 1964), i.e., the separation of granular and fibrillar elements, at concentrations not affecting cytoplasmic structures. The induction of nucleolar segregation by a chemical is therefore taken as an index of selective interaction with nucleolar DNA (nuDNA) (Simard, 1970).

Reynolds et al. (1963), has already shown that 4-NQO induces nucleolar segregation in cultured mammalian cells, suggesting that nuDNA is the target of 4-NQO. In this study we use Crithidia with its unique complement of organellar DNA, in an attempt to determine the selective binding properties of 4-NQO.

Part two of this thesis is predicated on an earlier finding that continuous exposure of a single-celled eukaryote to 4-NQO can induce

permanent defects in cell metabolism and morphology (Mita et al., 1966). Permanently altered clones such as these are considered to be microbial models of cancer cells (Gause, 1966]. Hutner et al. (1967) succeeded in inducing permanently bleached strains of Euglena gracilis by continuous exposure to 4-NQO; but Crithidia lacks an obvious morphological feature such as chloroplast development; hence, we turned to the well-characterized yeast respiratory deficient (RD) system for a suitable biochemical marker.(Nagai, 1961).

4-NQO has been shown to induce respiratory-deficient mutants in yeast independently by Nagai, (1969) and Epstein & St. Pierre, (1969) with efficiencies approaching 100%. The RD mutation constitutes a class of mutation characterized by alterations in (1) morphology (small colonies) and (2) physiology (slow growth) and biochemistry (alterations in respiratory pigments) (Nagai et al., 1961). It is particularly suitable for our purposes because both RD mutants and cancer cells have in common malfunctioning respiratory systems, hence the RD mutation has been considered by some as a yeast counterpart of cancer (Gause, 1966). The diagnostic techniques required to isolate RD mutants are readily applicable to Crithidia.

Our objective in Part two therefore, was to induce in Crithidia a respiratory-deficient condition, similar to that achieved in Saacharomyces, by continuous exposure to 4-NQO. The methods of diagnosis include the use of tetrazolium salts with which one can detect colonies deficient in electron transport (Ogur et al., 1957), measurement of growth rates, clone diameters, and spectrophotometric analysis of respiratory pigments (Chance, 1952; 1954).

In the following section the methods used in this thesis are described in detail. In section 3 and 4 results relating to the early changes induced by 4-NQO are presented and interpreted. In the 5th and 6th sections the permanent changes following persistant exposure to 4-NQO are presented and discussed.

METHODS

Organism and Conditions of Growth

Crithidia fasciculata ATCC 11745, obtained from S. H. Hutner of Haskins Laboratories at Pace College, New York, was maintained in screw-capped tubes containing 10 ml of defined or crude medium at 27 C. A laminar-flow hood was used to assure sterile conditions. Cultures for biochemical analyses were grown in 4 to 8 1-liter flasks containing 500 ml of growth medium. Crude medium contained 0.5% Trypticase, Baltimore Biological Laboratories (BBL), 0.5% yeast extract (BBL), and 1.5% sucrose, pH 7.6. Hemin (Sigma) was dissolved in 50% Triethanolamine added to a final concentration of 15 µg/ml before autoclaving. The doubling time was 3.3 hr (determined as time for doubling of absorbance at 540 nm measured in side-arm flasks (Bellco) between 0.1 and 0.7 absorbance units. The side arms fitted the carrier of the spectophotometer, enabling rapid measurements of turbidity after tipping the contents of the flask into the side-arm. The protozoa were grown to late log phase which usually required 2-3 days on a gyrorotary platform set in a water bath at 25 C.

The defined medium for Crithidia fasciculata was modified from that of Bacchi et al. (1969) by deleting guanosine, orotic acid, biopterin, and thymine, and substituting Triethanolamine for Quadrol as the hemin solvent; final pH 7.4 (see Appendix). The hemin in the low-heme medium had a defined medium final concentration of 2.0 µg/ml instead of 15 µg/ml, pH 7.2.

Cloning and Determination of Plating Efficiency

Early log-phase cells were counted by hemocytometer after immobilizing the cells with $HgCl_2$; appropriate culture dilutions for plating were made to obtain between 30 and 300 colonies per plate. One tenth ml of a cell suspension was added to each plate containing crude medium solidified with 1.25% agar ("Noble Agar", Difco) and spread with an "L"-shaped glass rod. Agar plates were prepared one day before use and checked for sterility. After 7-9 days growth was evident as colonies with a regular margin. Plating efficiency is expressed as the ratio of number of colonies counted to the number of cells plated X 100.

Tetrazolium Overlay Technique

Stock solutions were prepared as follows:

I. 0.67 M phosphate buffer pH 7.0. Triphenyl tetrazolium chloride (TTC) 0.06 M (0.2%).

II. Double-strength "crude" medium + 2.5% Noble agar.

Solutions I and II were sterilized by autoclaving and stored at 4 C. Tetrazolium reduction was tested by melting II and adding it to an equal amount of I. Approximately 10 to 20 ml of the final test agar (at 40-45 C) was poured over 7-9 day-old plates having 30-100 colonies. Within six hours of contact with the overlay, colonies endowed with intact respiratory chains reduced the colorless tetrazolium to the insoluble red formazan; colonies with a defective electron-transport system remained a translucent gray.

Preparation of Cells for Electron Microscopy

Cells grown in 10 ml of defined medium (in 125 X 20 mm loosely capped tubes) to a density of 7-8 X 10^6 cells/ml were exposed to 2.5 µg/ml 4-NQO for 1.25 hr at 27 C. These cells were centrifuged at 1,240 g, and washed 2X with RSM buffer. (RSM buffer, routinely used to preserve ribosomal integrity, was an excellent wash solution for intact cells to be fixed for electron microscopy.)

Electron Microscopy

Cells were fixed for 1 hr in 0.1 M sodium cacodylate buffer, pH 7.4, containing 2.5% glutaraldehyde and 0.05% calcium chloride. They were post-fixed in 1% osmic acid after washing in cacodylate buffer for 1 hr; these procedures were carried out at 4 C. Following fixation the pellet was stained in 1% uranyl acetate and dehydrated through a graded alcohol series. After clearing in propylene oxide the pellet was embedded in Epon (Luft, 1961) in polyethylene capsules. The capsules were placed in an oven at 60 C for 48 hr for polymerization. After polymerization blocks were removed from the capsules; and thin sections, cut with a Porter-Blum ultramicrotome equipped with a diamond knife, were picked up on uncoated copper grids. The sections were double-stained with uranyl acetate and lead citrate (Watson, 1958) and examined in a Phillips EM 300 electron microscope.

Ultrastructural Cytochemistry

Peroxidase staining (Graham & Karnovsky, 1966) as modified by Novikoff & Goldfischer (1969) was used to detect intracellular localization of catalase. This technique had allowed visualization of rat liver and rat kidney peroxisomes in aldelyde-fixed cells for both

light and electron microscopy; consequently, the procedure described under "Electron microscopy" was interrupted after the aldehyde fixation step. DAB oxidation medium (see Appendix) was then added after 2 washes in cacodylate buffer and the pellet incubated at 37 C for 0.5-1.0 hr. The reaction was performed at alkaline pH (pH 8-9) and high concentrations of DAB and H_2O_2 were used (see Appendix). After 2 washes in buffer, preparative procedures for electron microscopy were continued as described.

Cytochemistry

Protozoa were suspended in a buffer containing sucrose (0.25 M), tris (0.02 M), and bovine serum albumin (1%), pH 7.4. (Vickerman, 1962), a drop placed on a glass slide and air dried. The cells were fixed in calcium-formol fixation (see Appendix) for 5 min at 4 C (Pearse, 1968). After washing in the same buffer the slides were incubated in DAB oxidation medium pH 9 and 6 at 37 C for 1 hr.

The stained cells were washed in distilled water dried and photographed at 1250X under a Zeiss phase-contrast microscope.

Morphometry

Relative volumes of cell structures were measured on prints

enlarged to 8" X 10" at a final magnification of 19,500 diameters. A grid consisting of 17 parallel lines spaced 8.5 mm apart was laid over the electron micrograph; the grid was constructed from Scalfix Scales (Bell Art Products) ruled at mm intervals (see Fig. 16).

The length of the sampling line which traversed the cross section of each class of structures was measured in mm. Measurements on each of the parallel lines were tabulated separately as shown in Table 16. After the sum of individual columns was calculated, the ratio of each component to the whole cell was analyzed by multiplying the total for each componant by 100/ whole cell.

Two underlying assumptions of quantitative stereology are that the representative sections are randomly oriented and of negligible thickness (Loud, 1962, 1968; Weibel, 1969). A second person selected random sections to avoid bias of the investigator. Bias from section thickness was ruled out by Crithidia's being 5-8 μ in diameter whereas each section averaged between 0.03 and 0.06 μ. Although the dense bodies averaged 0.5 μ in diameter this factor is probably not significant with respect to section thickness (Baudhuin, 1968).

Preparation of Drug Solution

4-Nitroquinoline N-oxide recrystallized, m.p. 157-9 C (Beacon Chemical Ind.), was prepared under a hood by dissolving in water and warming gently on a hot plate equipped with a magnetic stirrer. A stock solution of 100 µg/ml was prepared and sterilized by filtration through a Swinnex-25 sterile filter unit (Millipore), pore diameter 0.22 µ. 4-NQO solutions were always prepared and stored in the dark to prevent photodynamic reaction in solution (Nagata et al., 1967).

Polarography

a) Preparation of Cells

Cells were grown aerobically to late log phase in an incubator equipped with a rotating platform. After the pH was raised from 4.5 to 7.6 with 1 N KOH, the organisms were centrifuged in 500 ml polypropylene bottles at 3000 rpm (1,465g) for 10 min in a Sorvall RC2-B refrigerated centrifuge equipped with a 6-place GSA fixed angle rotor and washed 2X in sucrose buffer. The protozoa were then pipetted into a 10 ml graduated conical centrifuge tube and centrifuged in a Hb-4 swinging bucket rotor at 2500 rpm(1,020 g) for 10 min. Excess hemin at the surface of the pellet was aspirated off. Suspensions

were quantitated as per cent packed cell volume (PCV); appropriate dilutions were made in sucrose buffer.

b) Measurement of O_2 Consumption

O_2 consumption was measured polarographically at 25 C in a Gilson Oxygraph (Gilson Medical Electronics, Model KM) equipped with a Clark-type electrode assembly. The electrode (Yellow Springs Instrument Co.), used at a polarizing voltage of 0.8, was inserted into a glass reaction chamber, of 6-ml capacity equipped with a magnetic stirrer. A Teflon-covered "flea" in the reaction chamber prevented sedimentation and unequal distribution of cells during the experiment and facilitated equilibration between the O_2 dissolved in the medium and that diffusing through the Mylar membrane covering the electrode. The final reaction mixture was 6 ml. The temperature was controlled by connecting the water-jacketed cell to a thermostated circulating bath.

Calculation of O_2 uptake was based on a normalized O_2 concentration of 240 µM at 25 C and ~760 mm Hg (Hill, 1968; Hill & White, 1968). Results are expressed as nmoles O_2/mg protein/min (Estabrook, 1967).

Protein Determination

Protein values were determined according to the method of Lowry et al. (1951). Bovine serum albumin (Sigma) served as standard. The Bausch & Lomb Spectronic 20 was used for all absorbance measurements.

Radioisotope Incorporation Experiments

Uracil-2-(^{14}C), specific activity 54.9 mC/mM (Amersham-Searle) was used to measure in vivo RNA synthesis. Side-arm flasks containing 100 ml defined medium were inoculated with Crithidia and grown to a density of 0.250 absorbance units (520 nm). Each flask received 0.2 ml of a 5 μC/ml solution of uracil-2-(^{14}C). One-ml aliquots were removed at various time intervals up to 30 min; 4-NQO was added and aliquots later removed at suitable intervals for 1.5 hr. Each aliquot was precipitated with cold 5% TCA, poured on a Millipore filter suction apparatus and washed 2X in cold TCA to remove acid-soluble material. The filters were oven dried, placed in vials containing toluene-base scintillation fluid, and counted in a Beckman liquid scintillation spectrometer (Paul et al., 1969).

Preparation of Crude Mitochondrial Fraction

Four 1-liter bottles of log-phase cells were harvested and the packed cell colume (PCV) determined. Acid-washed glass beads (200 μ, Minnesota Mining & Manufacturing), cells, and sucrose buffer were added to a Sorvall Omnimixer chamber in the proportions 1 gm:1 ml:2.5 ml; the cells were disrupted for 3-6 min at 7000 rpm, at which time examination of the slurry by phase-contrast microscopy revealed approximately 95% cell breakage. The homogenate was centrifuged at 2000 rpm (650 g) for 10 min to remove whole cells, large debris and glass beads. The resulting pellet was washed 2X with sucrose buffer and all supernatant fluids were pooled. A crude mitochondrial fraction was obtained by centrifuging at 10 X 10^3 rpm (16,318 g) for 10 min. The crude pellet was washed 2X in buffer and a small sample fixed overnight in 2.5% glutaraldehyde and prepared for electron microscopy as previously described. The crude mitochondrial pellet, consisting of mitochondrial fragments kinetoplasts and flagella, as observed under the electron microscope, was resuspended in 10-15 ml of sucrose buffer and used immediately for analysis of cytochrome spectra. All operations were at 4 C (see flow diagram).

Difference Spectra

The difference in absorption between reduced and oxidized suspensions of mitochondria was measured by a technique developed by Chance (1952, 1954) and modified for Crithidia by Hill et al. (1968). Spectra were obtained on a Cary Model 14 dual-beam recording spectrophotometer. The crude mitochondrial preparation was divided into equal portions, one of which was reduced by the addition of sodium dithionite and the other oxidized by aeration. The temperature was 25 C and the optical path length of the silica cuvettes 10 mm.

Catalase Assay

Protozoa were mechanically disrupted as described in "crude Mitochondrial preparation". After centrifuging at 2,500 rpm (1,020 g) to remove glass beads, whole cells and debris, the supernate was diluted 10-100 fold in sucrose buffer and assayed for total catalase activity. Determination of sedimentable i.e., microbody-bound catalase, was by centrifugation of 10,318 g supernate at 40,000 rpm (105,000 g) for 30 min in a Spinco model L-2 ultracentrifuge equipped with a ti-50 fixed angle rotor. The resulting pellet and supernate were

then assayed for catalase activity as follows (Diagram 3).

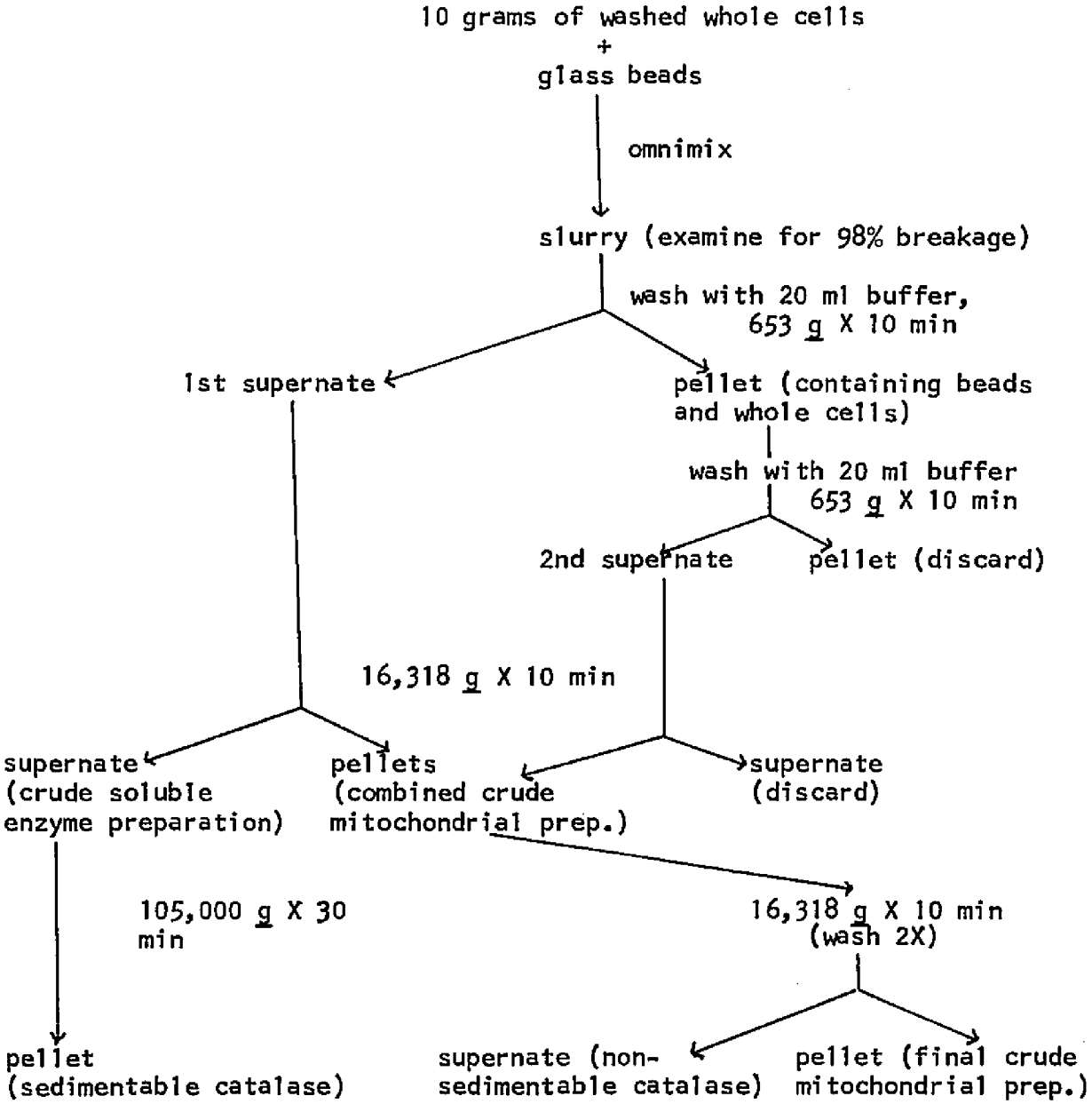
Preparations were pre-treated for 1 min with 1% v/v Triton X-100 (Baudhuin, 1968) to assure release of enzyme and were incubated at 0 C for 3 min in a mixture containing 0.2 M imidazole-HCl buffer pH 7.2, 0.1% bovine serum albumin and 0.0003% (1.5 mM) H_2O_2 (Baker) in a total volume of 5.2 ml. The reaction was stopped by addition of 3 ml of a saturated solution of titanium oxysulfate (K & K) in 2N H_2SO_4, diluted 1.5X. The remaining H_2O_2, converted into a yellow reaction product, was measured colorimetrically at 410 nm. The concentration of H_2O_2 at zero time was measured on an unincubated mixture of the same composition. One unit of activity is defined (Baudhuin, 1968) as the amount of enzyme causing the breakdown of 90% of the substrate in 1 min in a volume of 50 ml under the assay condions.

Measurement of Cell Size

Coulter-counter measurements were made on appropriate dilutions of Crithidia in an electrolyte containing 2% formaldehyde and 1.8% NaCl. Settings were adjusted to amplification 1/4, aperture current 1/8.

DIAGRAM 3

Flow chart for isolating Crithidia fasciculata crude mitochondrial and soluble fractions.



The lock position with lower threshold set at 5 and upper at 100 was used to obtain a frequency distribution of the volume of cells in the sample population in gradations of 10.

Statistical Methods

Mean ($\bar{x}$), standard deviation (SD), and standard error (SE) were computed separately for microbodies, mitochondria, and whole-cell volumes by averaging measurements obtained from individual cells. Testing the significance of the difference between the two means in each category was by the students t test (Dixon & Massey, 1969).

The method of least squares, as described in Dixon & Massey (1969) was applied to morphometric data. A scatter diagram was generated by plotting values for $\sum$ peroxisomes/whole cell in an XY plane. The regression line of Y on X calculated for mutant and control points were superimposed on the scatter diagram, and the significance of the difference between the slopes of the least square lines tested by the null hypothesis as described in Sokal & Rohlf (1969).

RESULTS

Part I

a) Standardization

1) Cloning and Freezing

Crithidia cells form colonies on crude or defined agar media. Although colony formation may occur with agar as high as 2%, plating efficiency declined sharply at agar concentrations above 2%; agar concentrations 1% resulted in a wet surface on which discrete colonies did not form. The optimum agar concentration was 1.25%. Colonies reached a maximum diameter of 1 mm at 7-10 days at 26 C. There was some heterogeneity in colony size; the larger colonies, occasionally seen, probably developed from a clumped cell mass. With surface spreading a reproducible plating efficiency of 50-60% was obtained (Table 1). Addition of different stabilizers including gum karaya, gum xanthan, and carboxymethyl cellulose did not improve plating efficiency.

A freeze-storage method, using glycerol, was developed in anticipation of isolation of mutant strains (Cohen & Zahalsky, 1969). Colony survival after freezing succeeded best when 5-7 day old colonies, grown on agar containing 5% glycerol, were overlaid with TTC agar. Glycerol concentrations above 5% sharply inhibited colony formation; at concentrations of 10 and 15% colony development was completely inhibited.

TABLE 1

Clonability of Crithidia. Cells were inoculated onto Petri dishes after appropriate dilutions and cell count. Colonies were counted on day 7. The culture contained ~7.95 X 10^6 cells/ml before dilutions in crude medium.

Number of organisms inoculated/plate	78	39	19
Number of colonies counted/plate	61	14	14
	61	27	15
	37	27	9
Average number of colonies/plate	53 ± 13.8	23 ± 7.5	13 ± 3.2
Plating efficiency (%)	67	61	60

After full color development (which normally occurred after 6-12 hr) plates were stored at liquid N_2 temperature (-74 C). After thawing at room temperature, red colonies were selected for isolation with a Pasteur pipette. Surprisingly, even in the absence of glycerol some colonies proved viable after inoculation into liquid media.

2) Polarography

Crithidia suspensions exhibit an average respiratory rate of 35.5 ± 4.3 nM O_2/min/mg protein over a 10-fold range of cell concentrations. As seen in Table 2, some variation above and below the mean respiratory rate occurs when expressed on a protein basis. However, it is clear that the rates are independent of the number of cells in the reaction chamber. A resuspended packed cell volume of 0.6%, in plotting O_2 consumption against cell number yielded a sharply rising slope in standard plots. This suspension was used in most experiments.

The effects of various known respiratory inhibitors on Crithidia is given in Table 3. Included in the table are results by Hill et al. (1968). Qualitatively the results agreed: DNP and rotenone had no effect, whereas cyanide, azide, and antimycin inhibited to varying degrees.

TABLE 2

Effect of Cell Concentration on Respiratory Rate in Crithidia. A PCV of 3 ml was diluted to a concentration of 3% (v/v) in sucrose buffer and dilutions ranging from 3.0 to 0.3% of the original suspension in the same buffer. Protein and cell counts were in duplicate on individual aliquots. Cells were equilibrated at 25 C for 30 min before measurements were made. Rates of respiration were calculated between 2 & 5 min to account for the initial burst of respiration which usually follows aeration of the cell suspension.

PCV (%)	Cell/ml X 10^6	µg protein/ml X 10^2	nm O_2/min	nm O_2/min/mg protein
0.3	41.2	4.12	76	30.8
0.6	82.0	8.80	227	43.1
0.9	123	14.3	304	35.5
1.2	164	18.9	372	33.6
1.5	206	21.6	480	37.0
3.0	412	33.5	656	32.8

However, significant quantitative differences were observed, i.e., Hill et al (1968) reported that 1 X 10^{-4} M NaN_3 inhibited respiration 68% compared to controls. In our experiments 5 X 10^{-5} M NaN_3 gave 100% inhibition.

b) Effect of 4-NQO

1) Microscopic Observations

Addition of 10-20 µg/ml 4-NQO at early log phase results in complete inhibition of growth. Within 15-30 min of exposure to 4-NQO most cells still possessed a limiting membrane and flagellum but were immobile; many were vacuolated. Approximately 20-30% of the cells in a given field were V-shaped, indicating arrested cytokinesis. A lance-like protuberance was noted in many cells which emerged laterally from the cell body distal to the flagellum. Occasionally ~~The numerous~~ rounded cells possessing two motile flagella and kinetoplasts, with one large nucleus in a much enlarged cell, were noted; this was also noted once in untreated cultures. The nucleus and kinetoplast appeared to have divided without subsequent cytokinesis.

LEGEND TABLE 3

Crithidia was grown for 48 hr in crude medium and harvested as described in "Methods". Cells were suspended overnight in sucrose buffer in order to eliminate non-motile cells, and then prepared for polarography. A suspension (1.5% PCV) was added to the reaction chamber and allowed to equilibrate at 25 C for 30 min. Inhibitors were added directly to the reaction chamber and the % inhibition was calculated as described by Estabrook (1967). Each inhibitor was tested 3X and the results averaged. At the end of each assay 1 ml aliquots were removed for protein determination and cell count. In the experiment the cell count was 23.2×10^7; protein, 1.5 mg.

TABLE 3

Summary of Effects of Respiratory Inhibitors on Intact Cells.

	Conc.[1] (M)	% Inhib.	Conc.[2] (M)	% Inhib.
NaN_3	1.0×10^{-5}	100[3]	1×10^{-4}	68
	5.0×10^{-5}	86		
NaCN	5×10^{-6}	85	5×10^{-4}	98
	5×10^{-5}	30		
Antimycin A				
	1×10^{-6}	100	1×10^{-4}	83
	0.5×10^{-5}	55.3		
2,4, DNP	5×10^{-3}	0	-	-
Rotenone	1×10^{-5}	0	-	-

[1]Results obtained by the author

[2]Results published by Hill et al. (1968)

[3]Based on average respiratory rate of 35.5 nm O_2/min/mg protein

2) Quantitation of Drug Effects

The IC_{50} is defined as that concentration of a toxic agent which reduces growth to helf that of controls as measured turbidimetrically in liquid media. As plotted in Chart 1, the IC_{50} of 4-NQO for Crithidia is ~2.5 µg/ml. The LC_{50} was determined by plating cells after exposure for 1 generation time (3.5 hr) and counting colonies after 7 days; Table 4 shows that the LC_{50} of NQO in undefined medium lies between 1 and 3 µg/ml.

3) Onset and Duration of Effects

To what extent could populations exposed to graded doses of 4-NQO recover viability over an extended time? A family of curves was obtained (Chart 2); each curve showed a different dose-response relationship. The greater the dose the longer the time required for resumption of growth. Surprisingly, up to a maximum tested dose of 5 µg/ml, growth was eventually restored to control levels. Another method of treating these data is a formulation in which the inhibition of cell growth by different concentrations of a drug is expressed by a straight line. In this way deviations from a theoretical line serves to evaluate the possible mechanisms of action of the inhibitor.

LEGEND CHART 1

Effect of 4-NQO on Growth of Crithidia in Liquid Medium. One ml aliquots from a 48-hr culture were inoculated into flasks containing 100 ml undefined medium and incubated at 27 C for 33 hr. 4-NQO was then added at final concentrations 1, 3, 5, 10, 15 µg/ml. Absorbance readings were taken at 33, 36.5, 51, and 67 hr after inoculation; the experiment was ended at 70 hr. The lines leading from the ordinate to the first experimental points are hypothetical ones drawn in on the basis of previous experiments. The pre-treatment values were not recorded because of the high reproducibility characteristic of the Crithidia growth cycle.

CHART 1

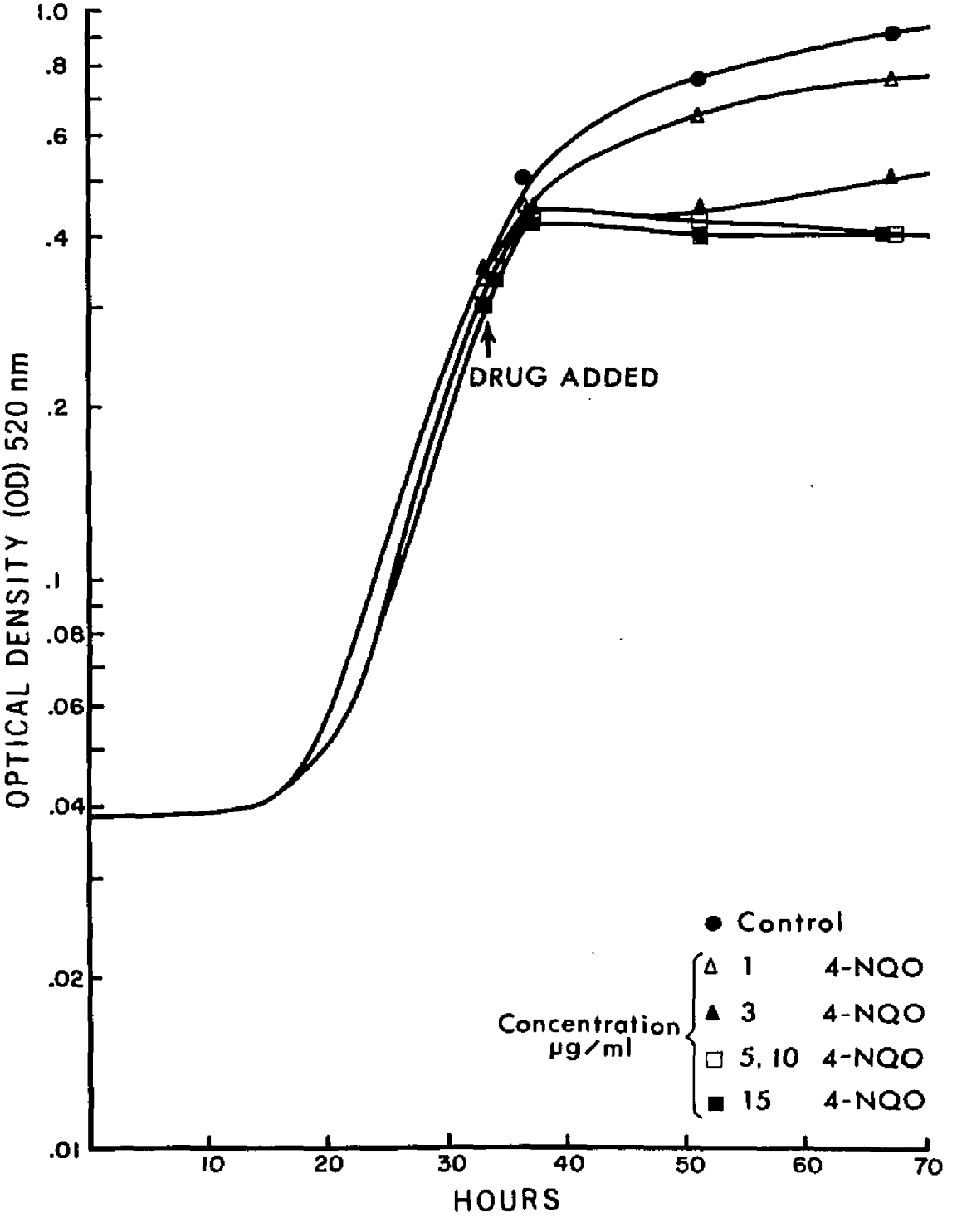

1.0
.8
.6
.4
.2
.1
.08
.06
.04
.02
.01
OPTICAL DENSITY (OD) 520 nm
DRUG ADDED
10
20
30
40
50
60
70
HOURS
● Control
Δ 1 4-NQO
▲ 3 4-NQO
□ 5, 10 4-NQO
■ 15 4-NQO
Concentration µg/ml

TABLE 4

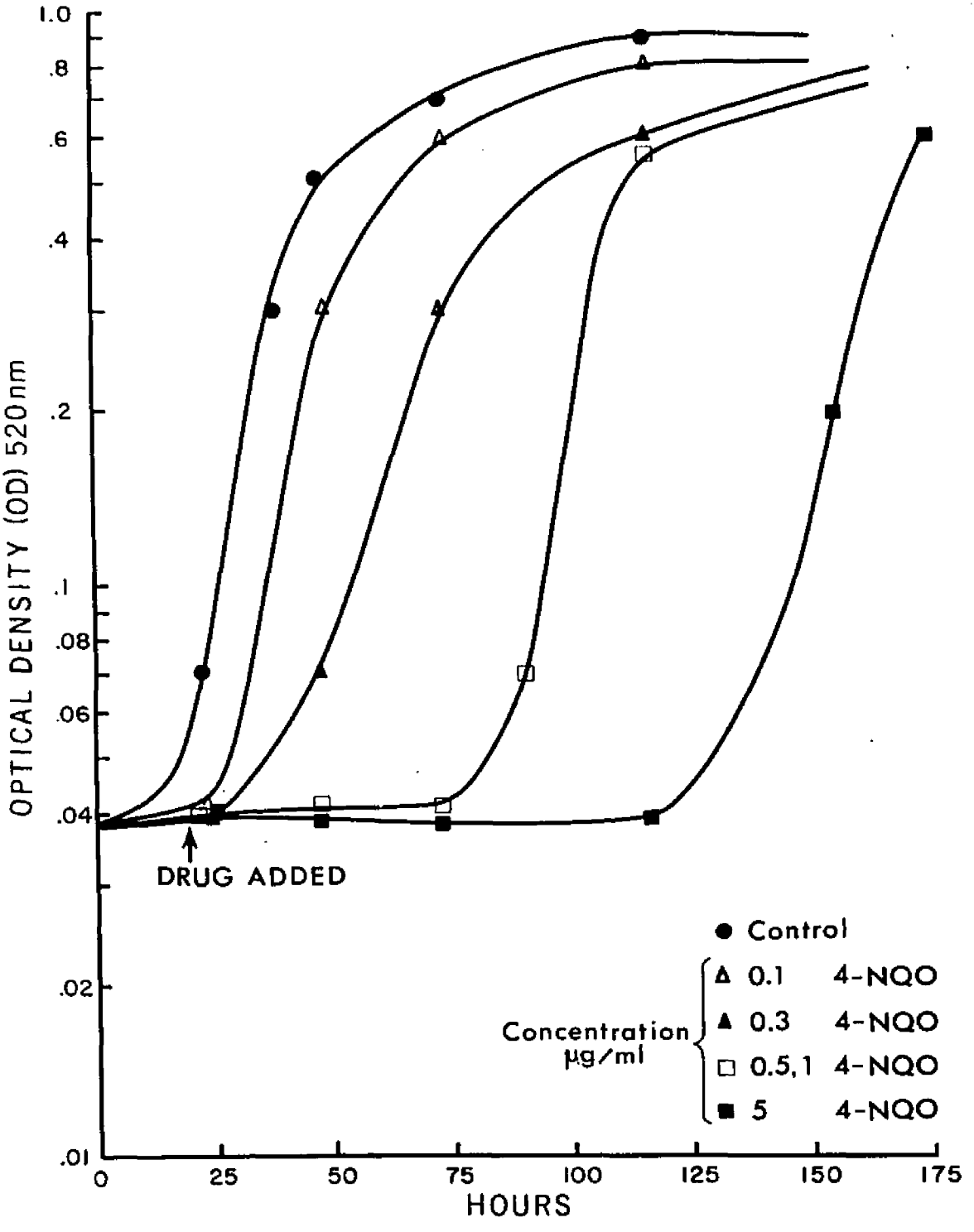
Determination of LC_{50} of 4-NQO. At 3.5 hr post-drug addition, cells from experiment described in Chart 1 were diluted in undefined medium and plated as described in "Methods". At day 9 colonies were counted.

Drug Concentration (μg/ml)	Colonies/plate	Average No. of Colonies/plate
0	790	840 ± 70.7
	890	
1	733	708 ± 35.4
	683	
3	29;26	24 ± 4.7
	23;18	
5	1;1	1
	0;0	
10	3;3;3;3	3
15	0;0;0;0	0

LEGEND CHART 2

Suppression by 4-NQO of Entry into Log Phase. One ml aliquots from a 48-hr culture of Crithidia were inoculated into flasks containing 100 ml undefined medium. At 10 hr 4-NQO was added to all but control flask at 0.1, 0.3, 0.5, 1, and 5 µg/ml final concentration. Absorbance measurements were made at 24, 47, 75, 150, and 175 hr post-inoculation and the results plotted on two-cycle semi-log paper.

CHART 2


OPTICAL DENSITY (OD) 520nm
1.0
.8
.6
.4
.2
.1
.08
.06
.04
.02
.01
DRUG ADDED
0
25
50
75
100
125
150
175
HOURS
Control
Concentration µg/ml
0.1 4-NQO
0.3 4-NQO
0.5,1 4-NQO
5 4-NQO

The formulation used is that derived by Schubert (1970):

$$y = ax + b$$

$$\log T_1 = \frac{\lambda(C)}{2.3} + \log T_{ext}$$

in which,

T_1 = net initial doubling time

or, $T_1 = T_a - T_o$

where:

T_a = doubling time in presence of inhibitor

T_o = doubling time in absence of inhibitor

C = concentration of inhibitor

T_{ext} = doubling time extrapolated to zero concentration

λ = a constant

and where $\frac{\lambda(C)}{2.3}$ and T_{ext} are the slope and intercept respectively of the straight line when T_1 is plotted against (C).

When the data taken from Chart 2 (see Table 5) were replotted on semi-log coordinates (Chart 3) a sharp deviation from linearity at concentration of ~1 µg/ml 4-NQO is seen. Because concentrations between 1 and 5 µg/ml 4-NQO were not used the exact nature of the

inflection point was not determined. The curve between 1 and 5 µg/ml was therefore hyperbolic. The experimental results conform to linearity up to 1 µg/ml and the line almost passes through the origin which conforms to theoretical expectations.

4) Influence of Growth Medium on the Cytotoxicity of 4-NQO

4-NQO forms complexes with certain amino acids and nucleobases (Nagata et al., 1963; Okano et al., 1969). Therefore it was important to assess the contribution of formation of such complexes with components in the medium in facilitating entry into the cell. Washed Crithidia was exposed to 4-NQO dissolved in H_2O. The results seen in Table 6 indicate that 4-NQO enters the cell without appreciable contributions from any component in the medium. The absence of cell survival in the IC_{50} dose may be attributed to the fact that determinations were made on a cell population of only 40×10^2 cells/ml instead of the usual density of $\sim 10 \times 10^6$ cells/ml. This emphasizes the importance of standardizing cell numbers in determining IC_{50}'s.

TABLE 5

Calculations from Chart 2 of Doubling Time at Varying Concentrations of 4-NQO

Inhibitor concentration	Time drug added (Abs. 0.04)	Time at doubling (Abs. 0.08)	Doubling time	T_1 Net doubling time
0	19 hr	25 hr	6 hr	0 hr
0.1	19	37	18	12
0.3	19	50	29	23
0.5-1.0	19	90	71	65
5	19	140	131	125

LEGEND CHART 3

Data from Table 5 Plotted with Semi-Log Coordinates.

CHART 3

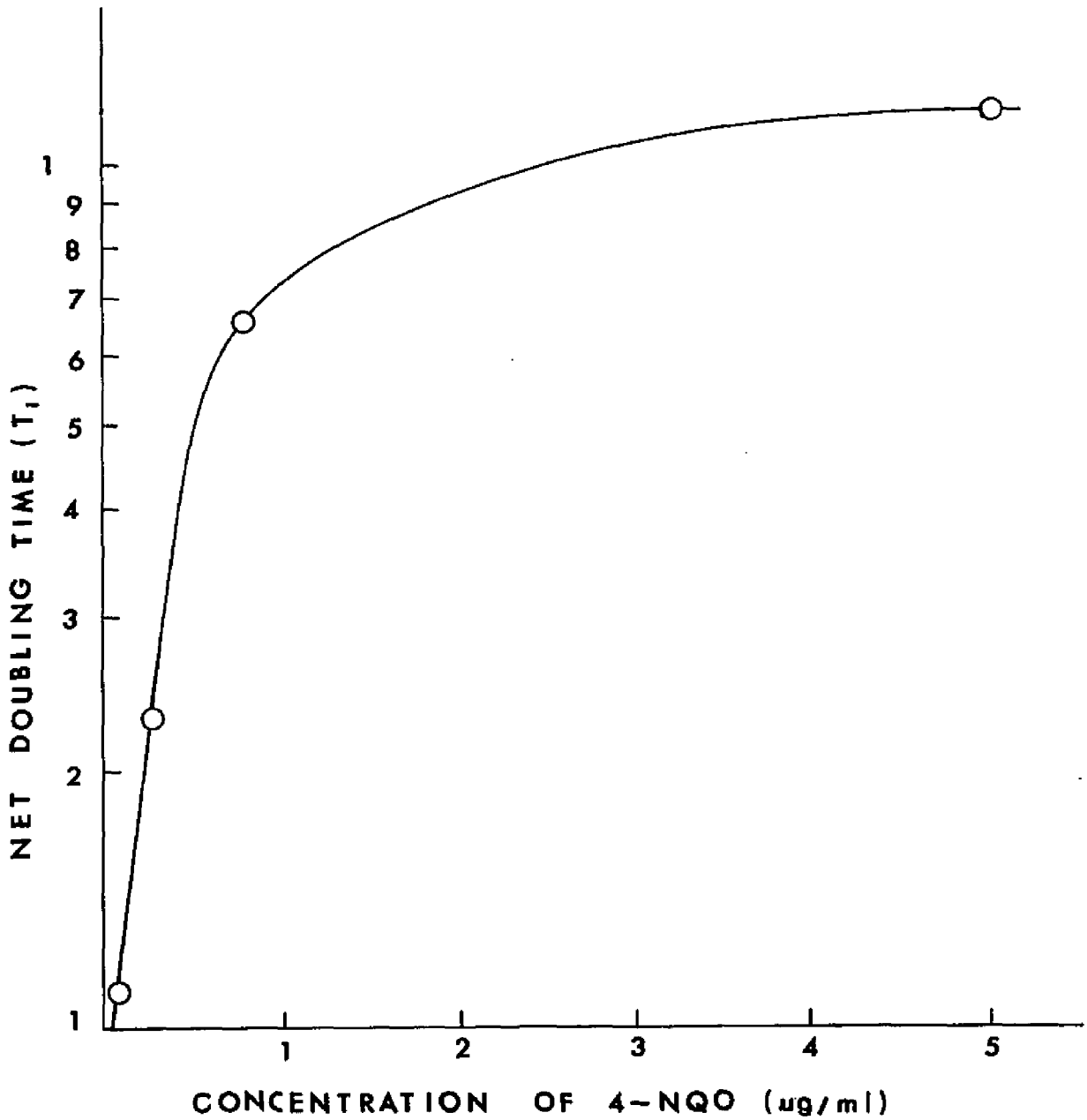

NET DOUBLING TIME (T_1)
1
9
8
7
6
5
4
3
2
1
1
2
3
4
5
CONCENTRATION OF 4-NQO (ug/ml)

TABLE 6

Influence of Growth Medium. Mid-log cultures were centrifuged and divided into two fractions; one fraction was diluted 10^{-4} in sterile distilled water; the other was diluted to the same concentration in undefined medium. 4-NQO at a concentration of 2.5 µg/ml was added to the experimental tubes for 30 min after which 0.1 ml aliquots were plated. Untreated cells were plated in the same manner. Colonies were counted on day 9.

	4-NQO in H_2O	Control	4-NQO in growth medium	Control
No. of Clones/plate	none	53;65 $\bar{X} = 59 \pm 8.5$	none	48;51 49.5 ± 2

5) Influence of Physiological State of Cells

The effect of 4-NQO on Crithidia at two points on the growth curve other than at mid-log is shown graphically in Chart 4 and in tabular form in Table 7. The two points chosen were at lag phase (time of inoculation) and at plateau phase. At these stages the populations are in grossly different physiological states. The data show that the effect of graded doses of 4-NQO added to a culture of Crithidia in early lag phase is complete inhibition at each concentration. The effect of similar doses of 4-NQO at stationary phase was measured by plate counts after 1, 2, and 7 hr of contact with 4-NQO. The results shown in Table 7 reveal that cells in plateau phase are sensitive to the lethal effects of 4-NQO to practically the same degree as cells in earlier phases of growth. In contrast to lag phase, both log and plateau phase cells showed a gradient of effect which varied as a direct function of drug concentration.

LEGEND CHART 4

Five large (250 ml) side-arm flasks containing undefined medium were inoculated with 2.5 ml aliquots of a 50 hr culture. 4-NQO, made up in undefined medium, was added at the time of inoculation at 1, 5, 10, and 15 µg/ml final concentration. The control flask was brought up to equal volume by the addition of an appropriate amount of undefined medium. At intervals of 5, 16, 24, 29, 40, and 50 hr absorbance measurements were taken and aliquots for cell count were removed. Incubation was at 27 C.

CHART 4

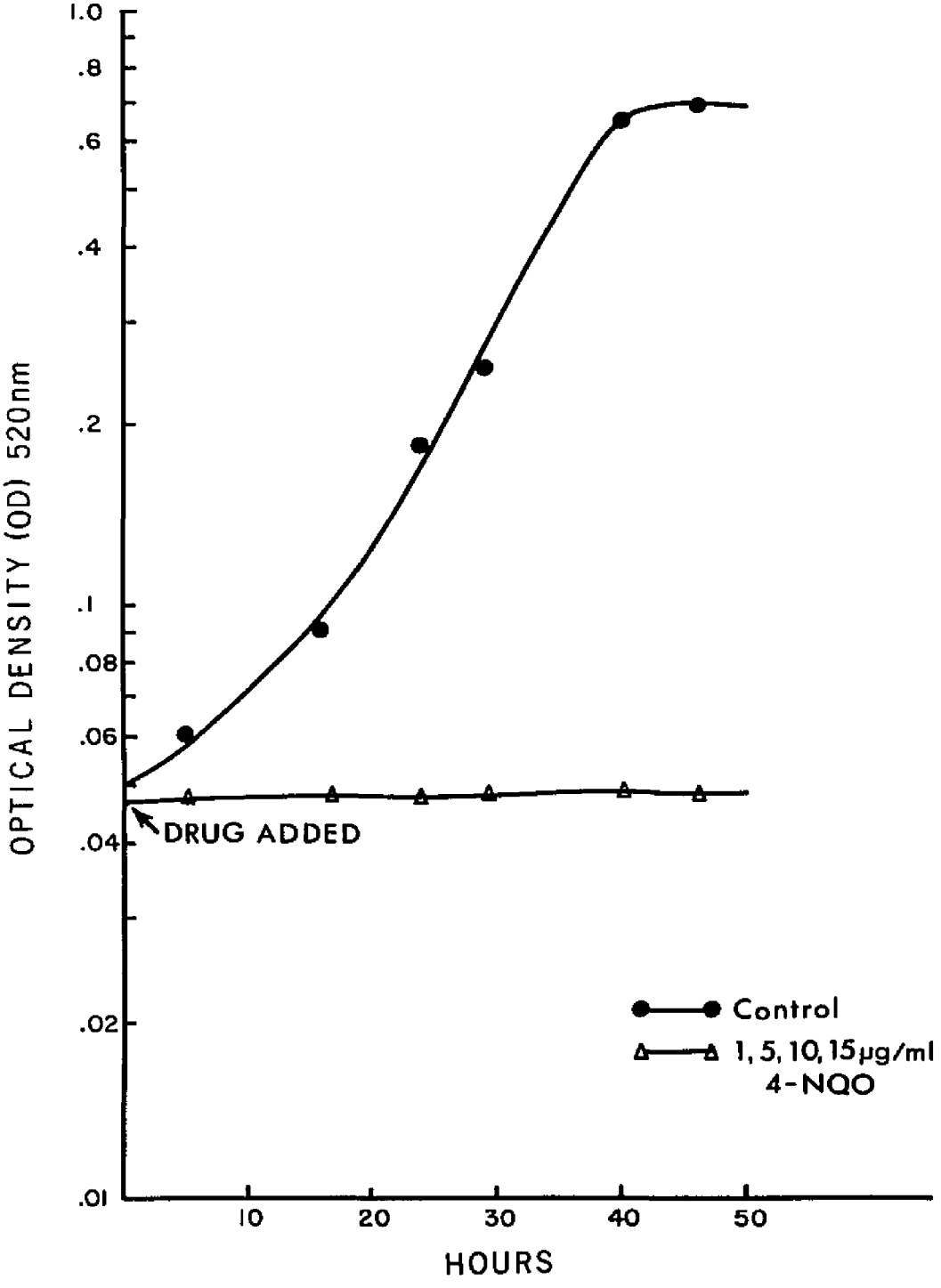

1.0
.8
.6
.4
.2
.1
.08
.06
.04
.02
.01
OPTICAL DENSITY (OD) 520nm
DRUG ADDED
Control
1, 5, 10, 15µg/ml
4-NQO
10
20
30
40
50
HOURS

TABLE 7

Effect of 4-NQO on Crithidia at Plateau Phase. Crithidia was grown for 2 days; at 50 hr graded doses of 4-NQO were added. At intervals of 1, 2, and 7 hr thereafter aliquots were removed, diluted to an appropriate concentration in undefined medium and plated. Counts were made on day 9. Values indicate the number of colonies counted per plate.

		Concentration of 4-NQO (µg/ml)			
		0	5	10	15
	0	400-500	→		
Time exposed to drug (hr)	1	↓	12;10	5;3	2;2
	2		70;14	3;1	2;0
	7		10; 8	1;0	2;0

6) Effects of 4-NQO on Crithidia Ultrastructure

Introductory remarks

The terminology used in this section requires a brief description of the principal structural components of the nucleolus. The terms used are those recommended by the Committee on Nucleolar Nomenclature (Waddington, 1966).

1) Granular or particulate component: 150-200 Å ribonucleoprotein particles, which are believed to contain 28S RNA

2) Fibrillar component: 50-80 Å fibrils, composed of ribonucleoprotein.

3) Amorphous component: a protein containing area of low electron density found within the nucleolonema.

4) Nucleolonema: threadlike elements 0.1 μ in diameter which are anastomosing and are arranged in either loose or compact masses.

5) DNA: autoradiographically shown to be diffusely scattered around the interior of the nucleolus; a tangled network probably containing the cistrons coding for rRNA.

The interphase nucleus of Crithidia appears oval, is centrally

placed, and shows a ruffled contour bounded by two unit membranes separated by a clear zone (Fig. 1, 2, 4). The interior of the nucleus is continuous with the cytoplasm through nuclear pores (Fig. 3). The outer membrane is occasionally studded with ribosomes (Fig. 4) and along the inner surface a faint aggregation of perichromatin material is often seen (Fig. 1, 4). Between the nucleolus and nucleolar membrane an area of low electron density is observed which contains chromatin. This chromatin containing region is not distinguishable in glutaraldehyde fixed cells.

The nucleolus is irregular in outline and has no membrane. It consists largely of nucleohistones, RNP fibrils, RNP granules and proteins (Busch, 1970). The substance of the nucleolus is made up of electron-dense granules about 150 Å in diameter; the granules resemble cytoplasmic ribosomes by virtue of their staining properties (Fig. 1, 2, 4, 8). These components are arranged in a loose reticular network, the nucleolonema. In addition, there are amorphous electron-lucid areas usually centrally located (Fig. 1, 4).

The kinetoplast is seen in the cytoplasm with anterior and

posterior extensions of the long single mitochondrion extending from it (Fig. 1). The kinetoplast of Crithidia is anterior and lateral to the nucleus and contains a central core of fine fibrillar material composed of DNA (Figs. 1, 8, 9). The fibrils stain strongly with uranyl acetate, fill the entire kinetoplast and lie parallel to the main axis of the organism (Figs. 1, 8, 9). Surrounding the central core is an electron dense matrix limited by two unit membranes approximately 100 Å thick (Figs. 1, 8).

Also in the cytoplasm is an organelle which has been described by Hill et al., (1968) as a dense body and by Brooker, (1971) as a bacilliform body but which on the basis of Rhodin's (1954) description (see below) we have designated as a microbody (Figs. 1, 2, 3, 4, 10, 12, 13, 14, 15) (also see Hruban & Rechcigl, 1969):

> "The location of the microbodies bears no relationship to the mitochondria, Golgi or nucleus. The number, size, and shape varies from cell to cell with a mean of about ten in each cell as counted by a section through the cell. The mean length is 0.3 μ and the mean width is 0.1 μ. They are surrounded by an osmium impregnated single membrane 45 Å thick. The ground substance of the microbodies exhibits the same opacity as the stroma in the mitochondria and consists of a similar finely granular structure, the size of the granules being about 40-50 Å."

Beneath the outer membrane are the sub-pellicular microtubules

(Fig. 3) (Angelopoulos, 1970; Messier, 1971) which are tubular in shape with a diameter of about 190-200 Å and wind a spiral course along the organism from anterior to posterior regions.

After treatment with 2.5 μg/ml (5.5×10^{-6}M) 4-NQO for 1.25 hr -- a rearrangement of the nucleolus into segments composed of aggregates of granules and fibrils of differing electron density -- was evident (Simard, 1970) (Figs. 3, 4, 5, 6, 7, 9). Moreover, there was an overall condensation and diminution of the nucleolar mass, with a consequent loss of the tangled reticular arrangement of the nucleolonema (Figs. 3, 5, 6, 7, 9). At least three components were seen: electron dense aggregates which appeared to be closely packed granules, a less dense region of coalesced fibrils almost equal in size (Figs. 3, 5, 6, 7, 9) and a smaller fragment, possible granular, that appeared to be migrating away from the central mass of the nucleolus (Figs. 6, 9). Sometimes, substances apparently derived from the nucleolonema were seen to lie at the periphery of a larger body comprised of tightly packed granules in a dense matrix (Figs. 5, 7). All nucleoli appeared oval in contour in contrast with the irregular contour typical of the

normal nucleolus and appeared to have shifted to the center of the nucleus.

When three nucleolar regions were evident a distinct polarization was seen with the largest region at one pole and the smallest at the opposite pole (Fig. 7). In some sections only a condensed remnant of closely spaced fibrils and some nucleolar fragments remained (Fig. 9). In no sections were amorphous regions observed. Particles, probably corresponding to nuclear inclusions, were found in several sections (Fig. 7). No conspicuous reproducible, ultrastructural changes were detected in the cytoplasm.

The kinetoplast of 4-NQO treated cells was of normal size and structure. The DNA-containing fibrils remained attached to the inner membrane and appeared loosely packed due to the oblique plane of section (Fig. 9). Examination of large numbers of sections showed normal kinetoplasts in practically all cases. Characteristic mitochondria were also present (Fig. 3); recognition of specific changes in the cristae such as swelling and orientation was subjective and these changes did not occur in all cells.

Cells treated with the non-carcinogenic derivative AQO were indistinguishable from control cells (Fig. 2, 8); no changes were seen in either the nucleus or cytoplasm. The nucleoli of AQO treated cells exhibited no fragmentation or diminution in size and were typically irregular in contour.

7) Biochemical Studies

Incorporation of uracil-2-C^{14} into acid insoluble material (sedimentable at 100,000 g for 1 hr) was linear during the first hour following exposure to label (Chart 5).

The addition of 4-NQO at concentrations of 1, 2.5, and 5 µg/ml resulted in an immediate cessation of incorporation (Chart 5); moreover, for a period of two hr after initial exposure to isotope an appreciable degradation of previously labeled RNA occurred. At two hours treated cells retained only 60% of label incorporation during the 30 min prior to drug addition. The rate of degradation of previously labeled material gradually diminished and approached an asymptope at approximately two hr. Inhibition of incorporation of uracil-2-C^{14} into acid precipitable RNA was complete after 60 min of exposure to graded doses of 4-NQO.

SYMBOLS FOR FIGURE LEGENDS

cr, cristae; f, fibrillar component of the nucleolus; fl, flagellum; fp, flagellar pocket; g, granular component of the nucleolus; i, intranuclear granules; K, kinetoplast; lg, lamellar Golgi; M, mitochondria; mi, microbody; N, nucleus; Nm, nuclear membrane; n, nucleolus; nf, nucleolar fragment; Ni, intranuclear inclusion body; Np, nuclearpore; ns, nucleolar segregation; r, ribosomes; t, sub-pellicular microtubule; v, vesicle; vg, vesicular Golgi.

Fig. 1. Longitudinal section through untreated Crithidia grown in defined medium and fixed and stained after 24 hr. Note the irregularly shaped nucleolus (n) composed of granular (g) and fibrillar (f) elements located within the nucleus (N). The nucleus is delimited from the cytoplasm by a double nuclear membrane (Nm). Located in the cytoplasm are the single membrane bounded electron-dense, microbodies (mi), the kinetoplast (K) lateral and anterior to the nucleus and its associated mitochondrion (M). Scattered through the cytoplasm are sections cut at random through the long single mitochondrion in which cristae (cr) are apparent. Also in the cytoplasm is a well-developed Golgi apparatus (1g) often located adjacent to the flageller pocket (fp). Numerous cytoplasmic vacuoles (v) of unknown function also are found in the cytoplasm, along with abundant free cytoplasmic ribosomes (r). Note the relative paucity of endoplasmic reticulum. X 25, 060

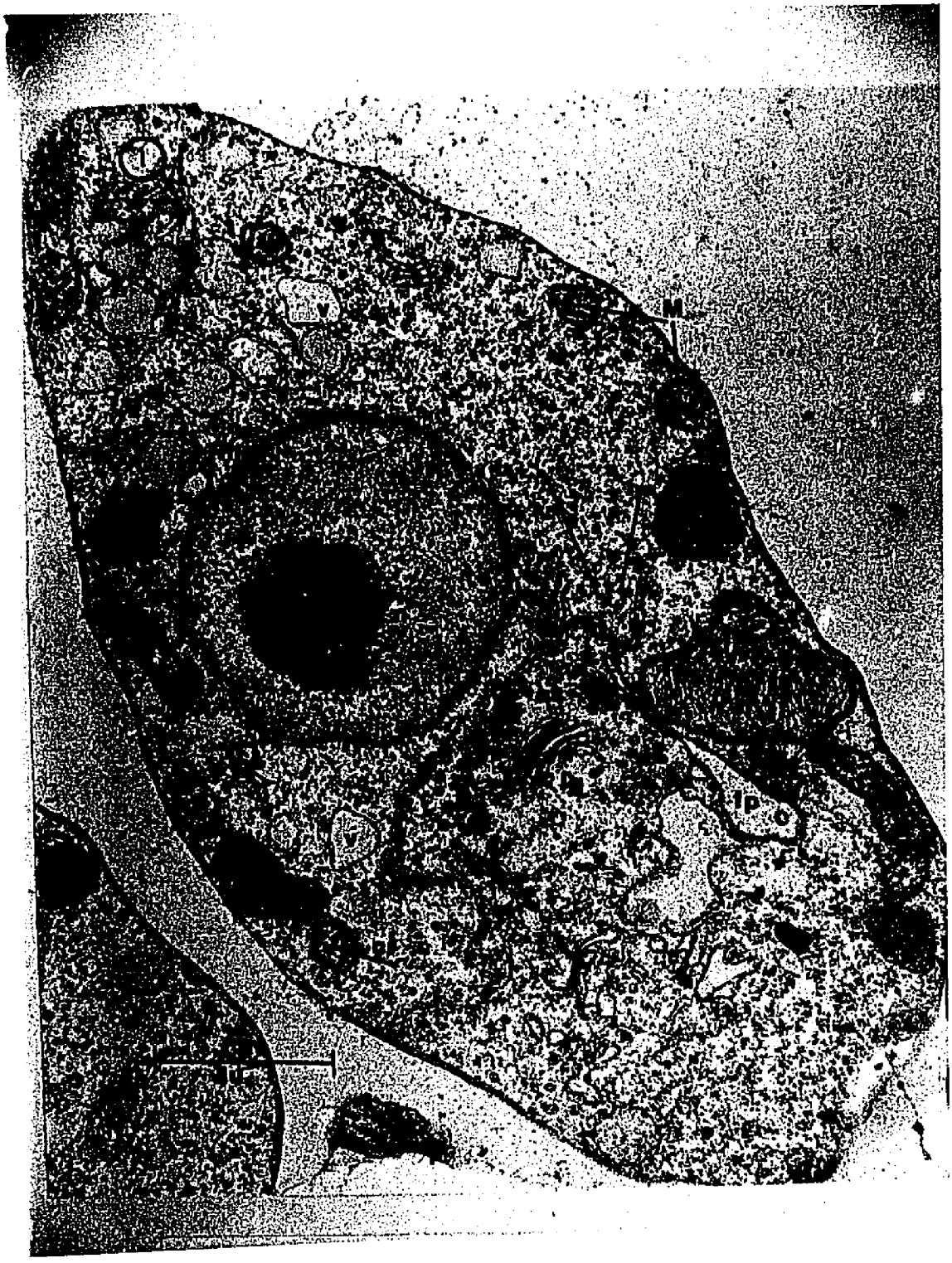

M
V
V
Ip
C

Fig. 2. Transverse section through Crithidia treated with 2.5 µg/ml 4-AQO for 1.25 hr. Note the normal appearance of the nucleolus (n) with its skein-like arrangement of granular (g) and fibriller (f) elements. No significant morphological alterations can be seen in either the nucleus (N) or cytoplasm of these cells. X 25, 080

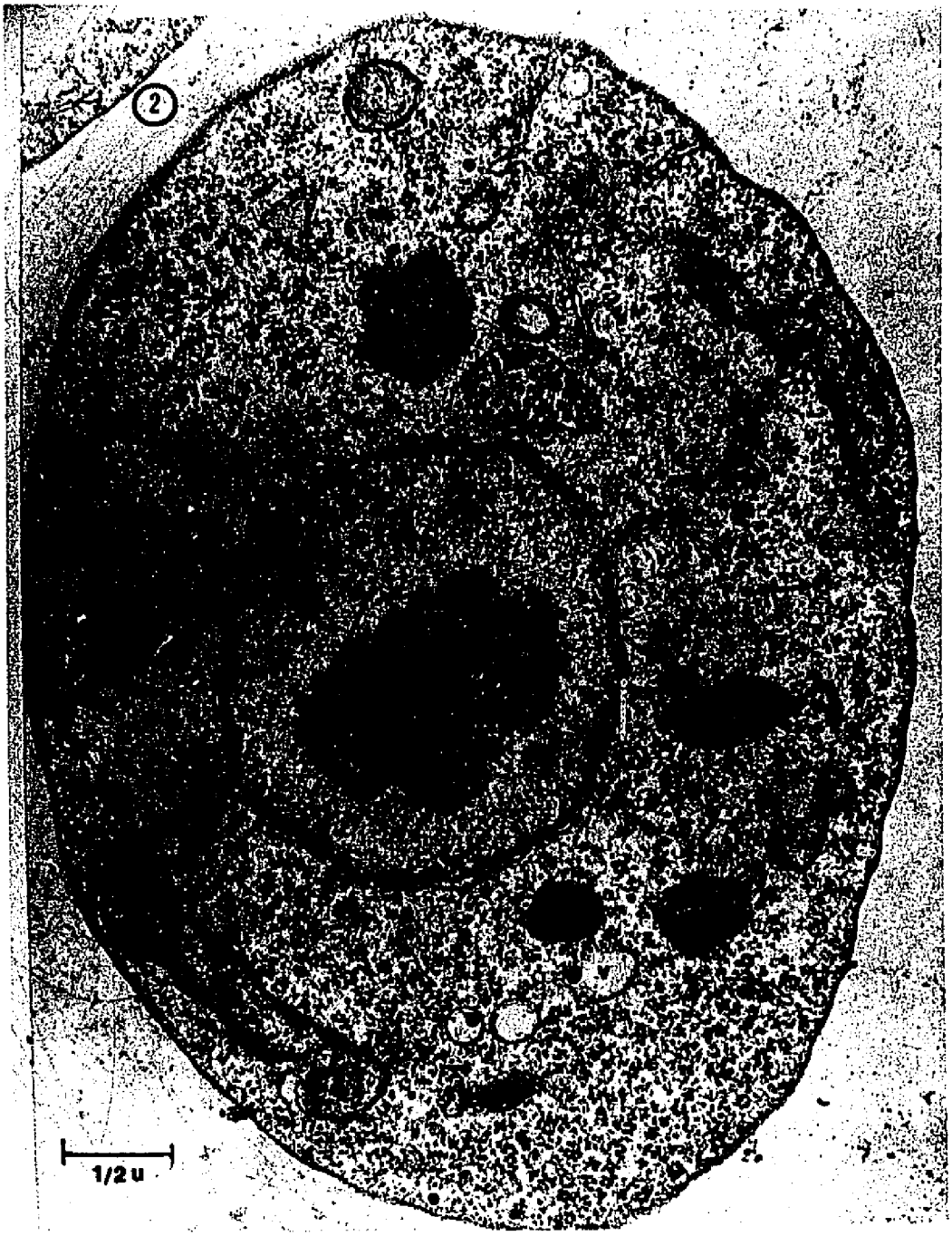

2
1/2 u

Fig. 3. Transverse section through Crithidia treated with 2.5 µg/ml 4-NQO for 1.25 hr. Note the redistribution of nucleolar materials into two clearly defined areas of differing electron density and the loss of the normal skein-like appearance (ns). In this section a nuclear pore (Np) can be seen which, though not shown, is present in normal cells. Also in the nucleus are intranuclear granules (i) resembling the granular components of the nucleolus. The cytoplasm appears normal with no diminution in free cytoplasmic ribosomes (r) or alterations in the Golgi (vg) apparatus, microbodies (mi) of mitochondria (M). The section through the mitochondria at top right appears slightly swollen butthis was rarely seen. In this section the flagellum (fl) can be seen lying within the lumen of the flagellar pocket (fp). Beneath the cell membrane are the sub-pellicular microtubules (t) seen in transverse section. X 38,300

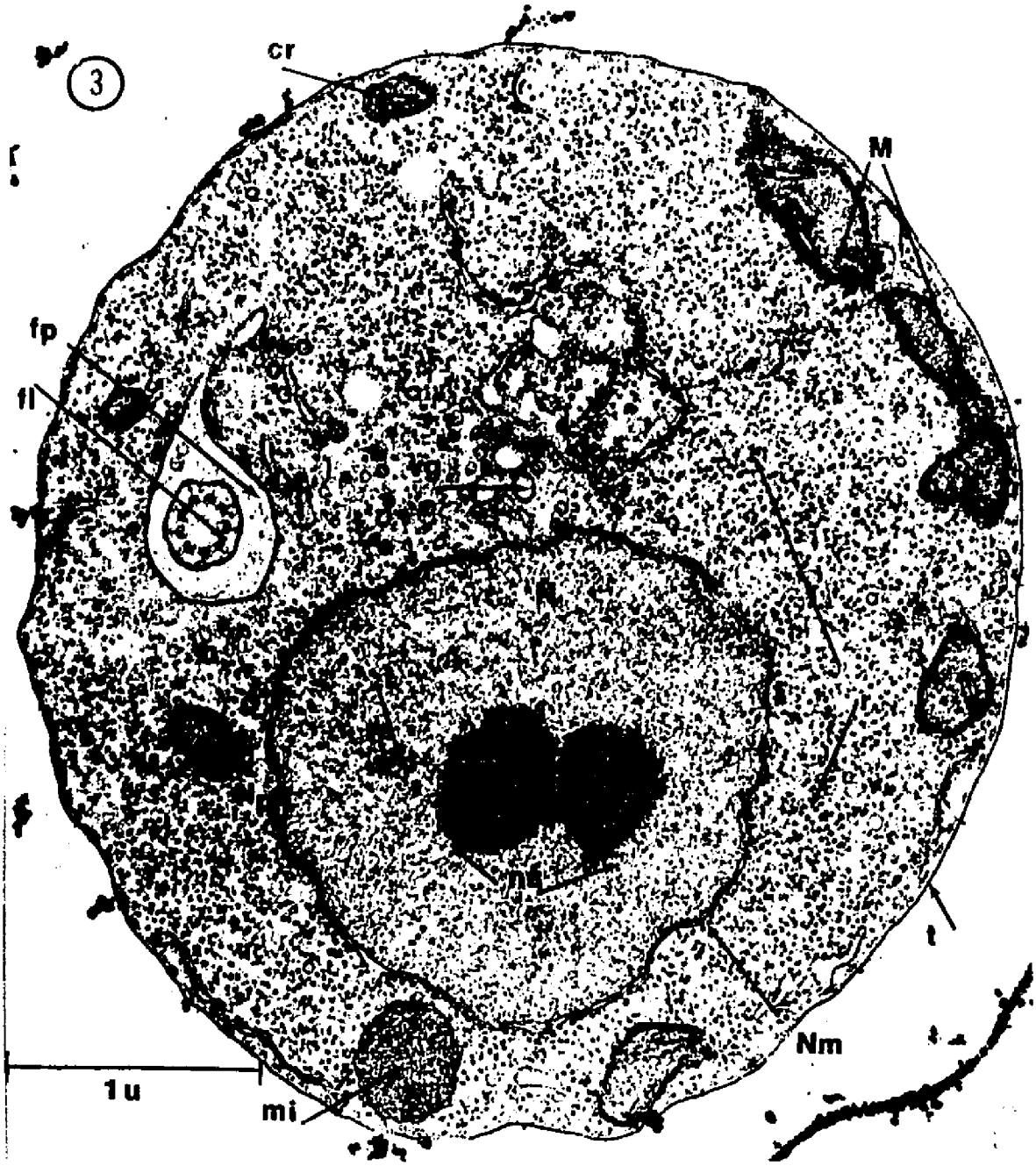

3
cr
M
fp
fl
nl
t
Nm
1u
mi

Fig. 4. The nucleus of untreated Crithidia grown in undefined medium, fixed and stained after 24 hr growth. Note the arrangement of the granular (g) and fibrillar (f) elements of the nucleolus. The granular component appears to be concentrated peripherally while the fibriller is centrally located. A microbody can be seen at top right (mi). X 36,000

Fig. 5. A section through the nucleus (N) of Crithidia treated with 2.5 µg/ml 4-NQO for 1.25 hr. Note the segregation of nucleolar elements into two masses of different electron densities (ns). A marginal condensation of electron dense particles can be seen adjacent to the inner nuclear membrane and the nuclear matrix appears less electron opaque. X 30,600

Fig. 6. A section through the nucleus of Crithidia treated as in Fig. 5. Note the segregation of nucleolar components (ns) and a nucleolar fragment (nf) which may represent ribonucleoprotein in transit to the cytoplasm. X 31, 400

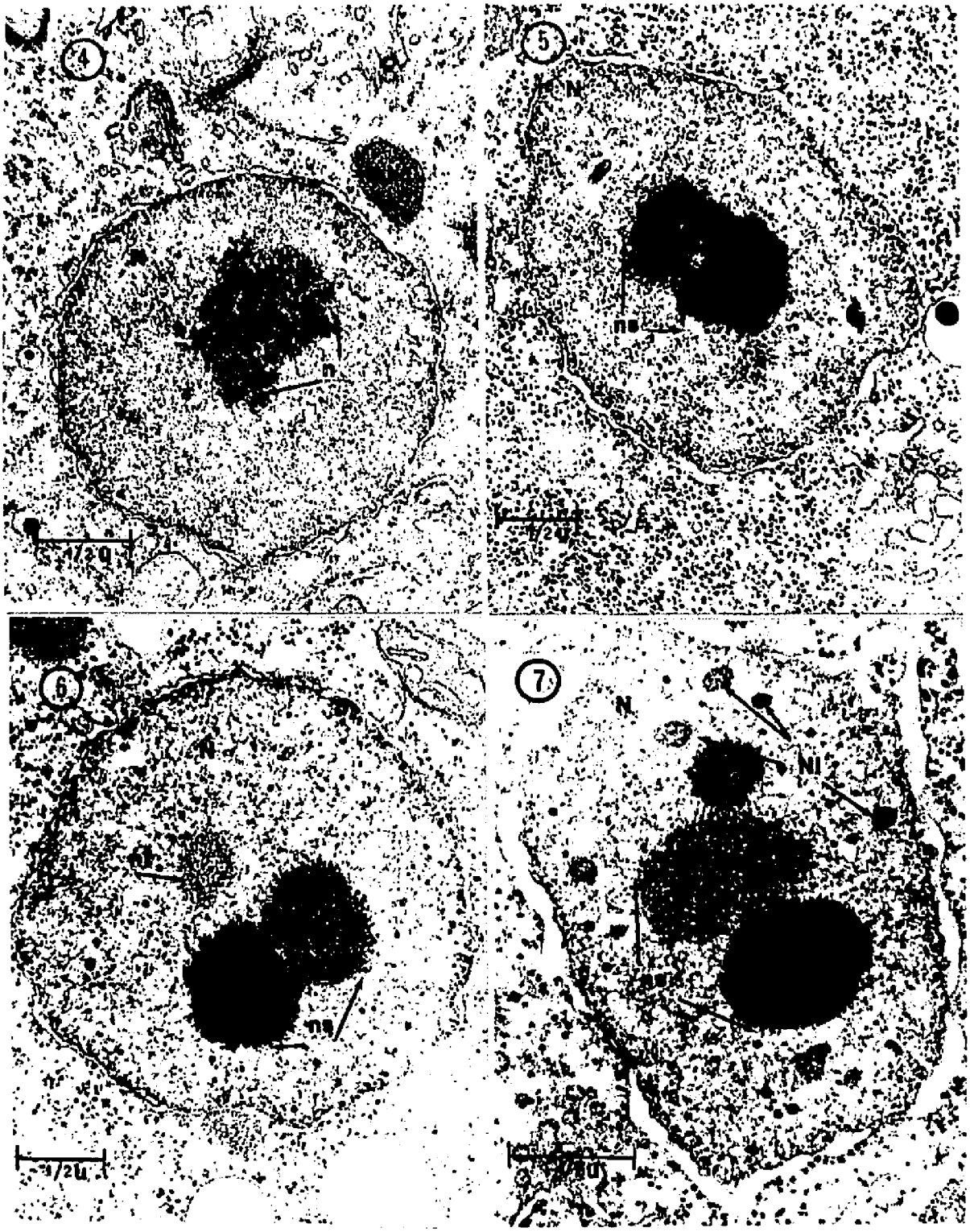
Fig. 7. A section through Crithidia treated as described in Fig. 5. Here a more complex form of nucleolar segregation is seen showing division into three unequal segments (ns) with a definite polarity from top to bottom, with respect to both size and electron density. Note the crescent-shaped ring of electron dense material in the lowermost segment partially surrounding a lighter central core. Also shown are particles, whose origins are unknown, which may be intranuclear inclusion bodies (Ni). X 44, 280

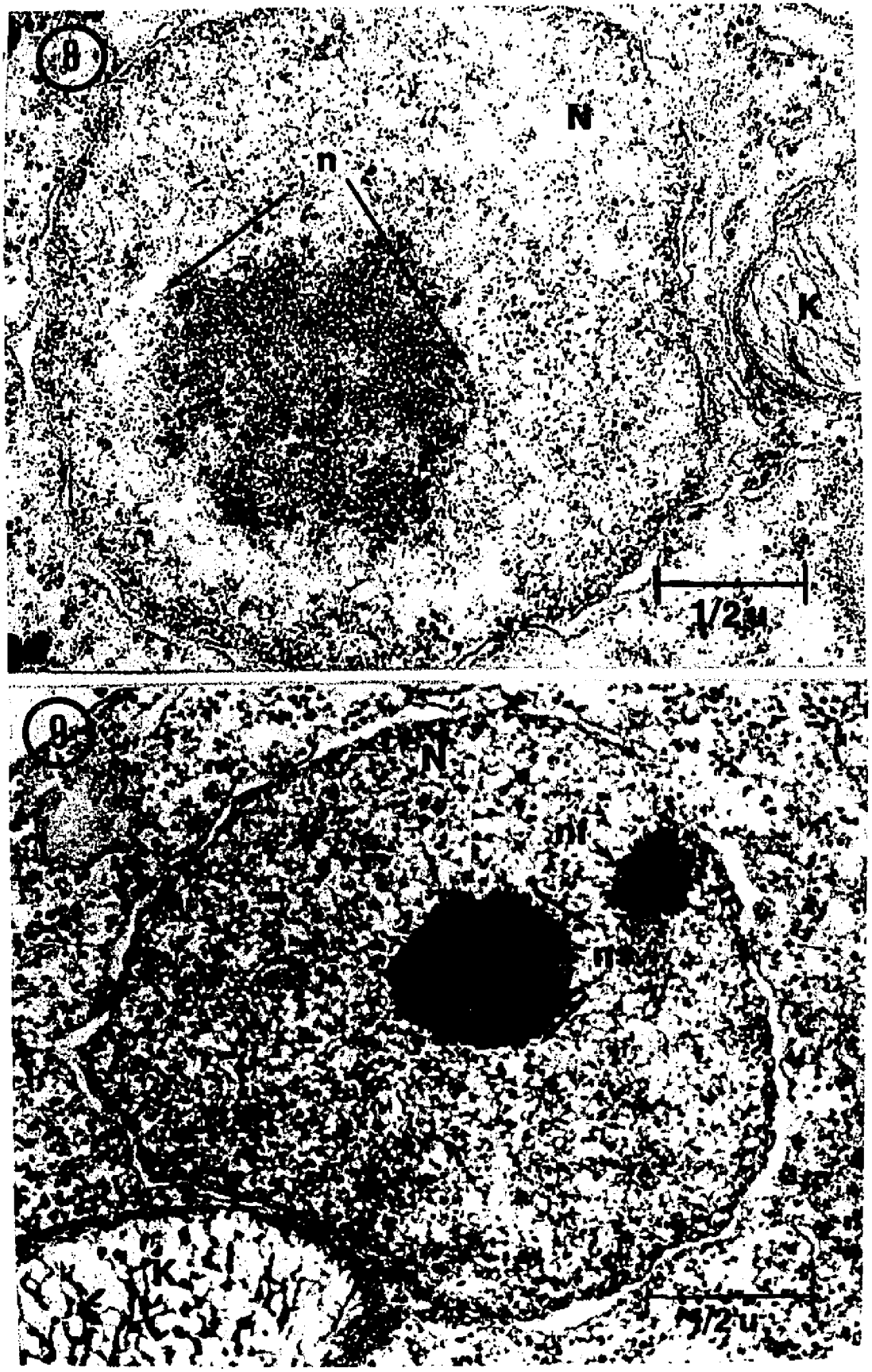
Fig. 8. Section through the nucleus of Crithidia treated with 2.5 µg/ml 4-AQO for 1.25 hr. Note the typical arrangement of the nucleolar elements characteristic of the normal nucleolus. The granular (g) and fibrillar (f) elements tend to occupy peripheral and central positions respectively. Note also the kinetoplast (K) lateral to the nucleus (N) with its double membrane and kDNA in oblique section. X 49,700

Fig. 9. A section through the nucleus of Crithidia treated with 2.5 µg/ml 4-NQO for 1.25 hr. Note the diminution in size of the nucleolus (ns) and its movement to a more central position in the nucleus (N). A

nucleolar fragment (nf) can also be seen adjacent to the nuclear membrane. The kinetoplast is seen in transverse section (K).

X 61, 200


4
n
1/2 u
5
N
ns
1/2 u
6
N
ns
1/2 u
7
N
Nl


8
N
n
K
1/2μ
9
N
n
n
K
1/2μ

The effect of exposure to 4-AQO on incorporation of radioactive uracil into Crithidia RNA contrasts sharply with that of 4-NQO. It can be seen graphically in Chart 6 that 4-AQO has no effect on the uptake of label. The experiment was designed to be internally consistent. 4-NQO was added after 30 min of exposure to radioactive label thus enabling comparison between pre and post 4-NQO rates of incorporation.

8) Physiological Effects of Exposure to 4-NQO

Impairment of Plating Efficiency

The plating efficiency of cells exposed to 4-NQO was dramatically impaired (see Chart 7). Most striking was the time of onset of drug action. Within the first minute of exposure cloning capacity was reduced by 56%; by four minutes it was almost zero.

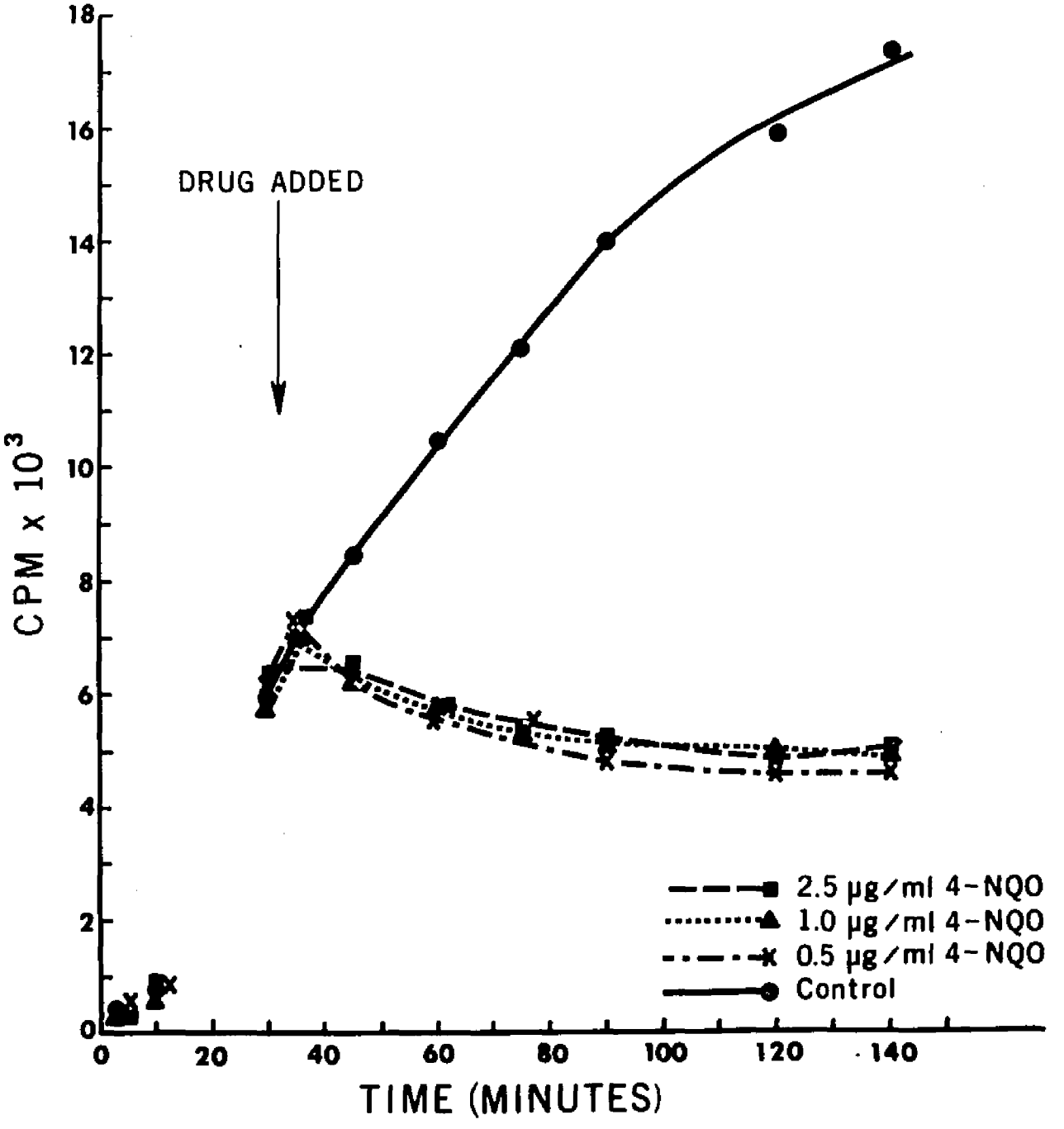
The failure of AQO to impair cloning efficiency at concentrations of 5 and 10 µg/ml over a time interval of 45 min is shown in Table 8. From the data prescribed it is not possible to determine whether 4-AQO permeates the cell boundary but has no toxic effect or is blocked at the cell membrane.

LEGEND CHART 5

Effect of 4-NQO on Uptake of Uracil-2-C^{14} in TCA Insoluble Material.

Four side-arm flasks containing 100 ml defined media were inoculated with 1 ml of a 48 hr culture and incubated at 27 C until turbidity reached 0.2 to 0.3 OD units. Then 0.2 ml of a 5 µC/ml solution of uracil-2-C^{14} was added, and 1 ml aliquots removed at the following time intervals: 3, 10, 30 min. At 32 min 4-NQO was added to 3 different flasks at final concentrations of 2.5, 5 and 8 µg/ml and 1 ml aliquots taken at 35, 45, 60, 75, 90, and 150 min. Each aliquot was pipetted into 3 ml cold 5% TCA and treated as described in "Methods". All aliquots were taken in duplicate.

CHART 5

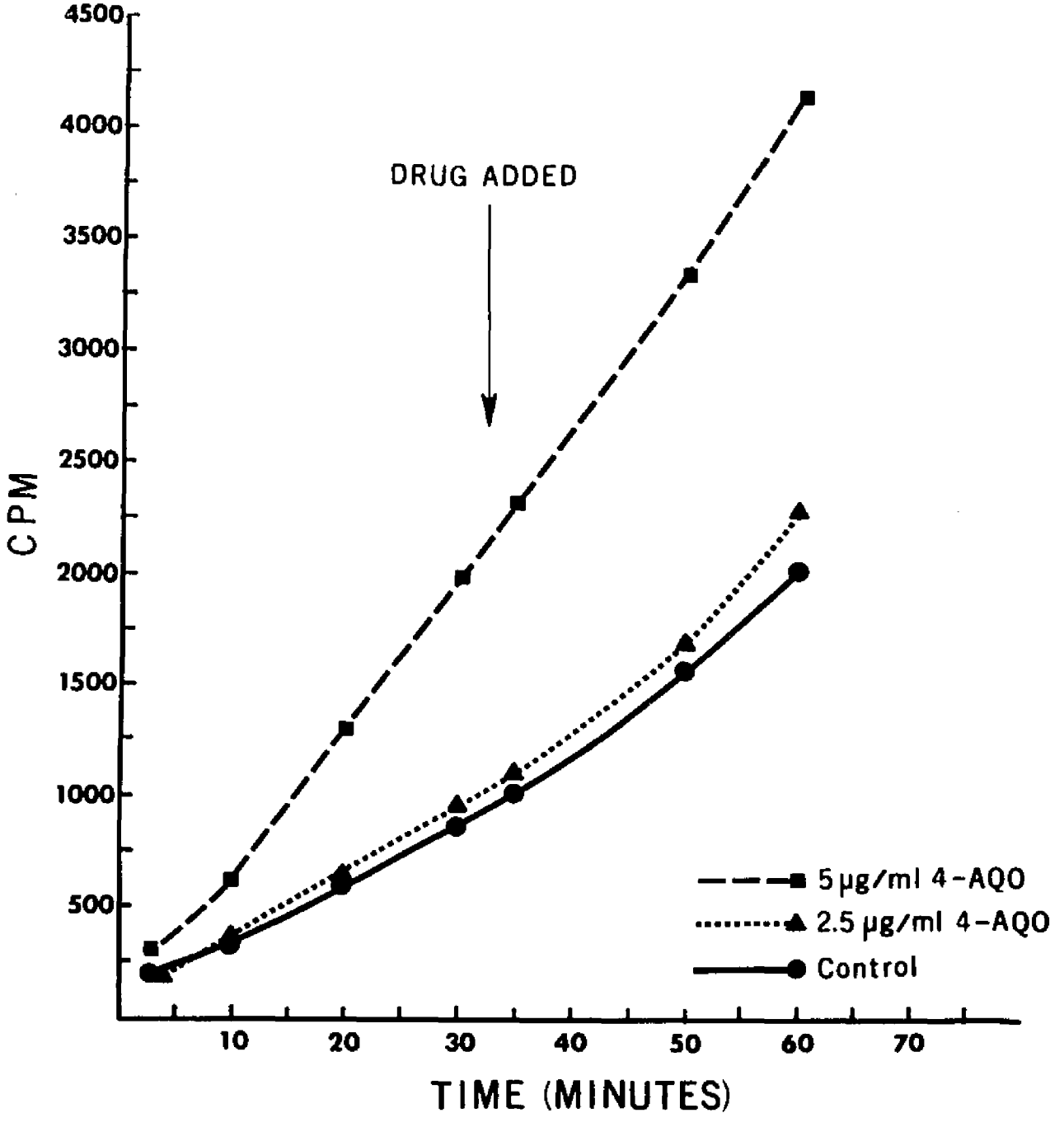

CPM x 10^3
18
16
14
12
10
8
6
4
2
0
DRUG ADDED
0
20
40
60
80
100
120
140
TIME (MINUTES)
2.5 µg/ml 4-NQO
1.0 µg/ml 4-NQO
0.5 µg/ml 4-NQO
Control

LEGEND CHART 6

Effect of 4-AQO on Uptake of Uracil-2-C^{14} in TCA Insoluble Material.

Experimental design described in Legend Chart 5. At 33 min after addition of uracil-2-C^{14}, 4-AQO at concentrations of 2.5 and 5 µg/ml were added and aliquots taken at 35, 50, and 60 min. Duplicate aliquots were taken at each time point.

CHART 6


4500
4000
3500
3000
2500
2000
1500
1000
500
CPM
DRUG ADDED
5 µg/ml 4-AQO
2.5 µg/ml 4-AQO
Control
10
20
30
40
50
60
70
TIME (MINUTES)

LEGEND CHART 7

Effect of 4-NQO on Cloning. Cells grown to mid-log in undefined medium in 10 ml screw cap tubes. 4-NQO added at 2.5 µg/ml, aliquots removed at 1, 2, 3, 4 min, diluted one thousand fold (10^3) and spread on agar plates as described in "Methods". Clones were counted on day 9. Each value is the average of five plates.

CHART 7

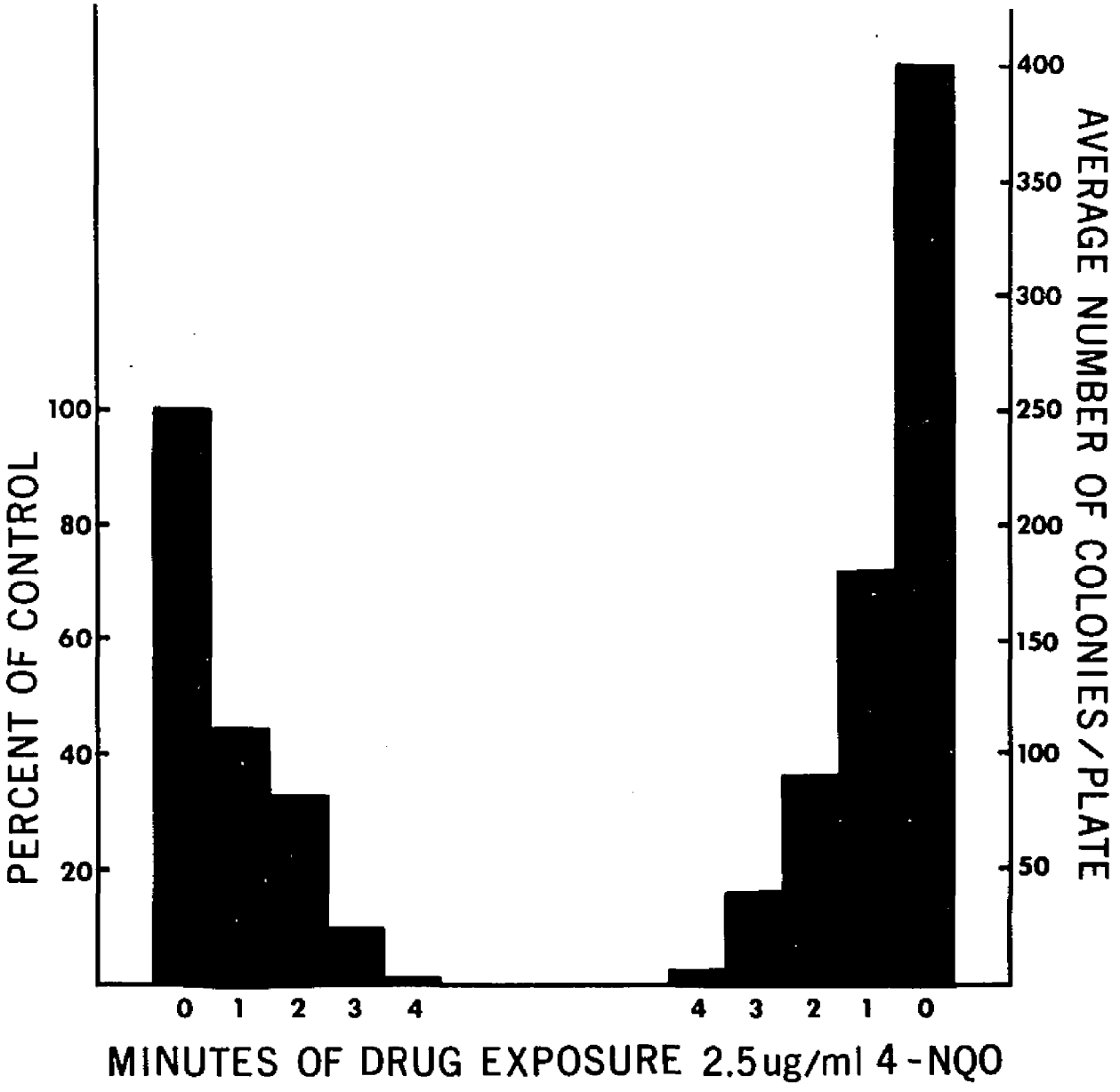

PERCENT OF CONTROL
100
80
60
40
20
AVERAGE NUMBER OF COLONIES/PLATE
400
350
300
250
200
150
100
50
0 1 2 3 4
4 3 2 1 0
MINUTES OF DRUG EXPOSURE 2.5ug/ml 4-NQO

TABLE 8

Effect of AQO on Cloning. One-tenth ml of a 40 hr culture of Crithidia was inoculated into 10 ml of undefined medium and incubated for 24 hr at 27 C. 4-AQO was then added at 5 and 10 µg/ml and the mixture was incubated for 5 min at which time 0.1 ml aliquots were plated after appropriate dilution; the process was repeated at 15, 25, 45, and 60 min. Three plates were inoculated at each time point. Colony counts were done on day 8.

	Minutes Post-Drug Exposure				
Concentration µg/ml	0'	5'	15'	25'	45'
	No. Colonies/plate				
5	120	114	200	98	169
	106	110	187	120	51
	180	184	143	108	184
(Mean) SD	135 ± 39	136 ± 41.6	176 ± 29.8	108 ± 10.9	135 ± 72.8
10	137	113	182	180	94
	110	100	120	138	106
	100	102	63	125	75
(Mean) SD	116 ± 19	108 ± 5.7	121 ± 59.5	147 ± 28.7	92 ± 15.6

Inhibition of Respiration

Cell respiration was reduced to about one-half that of controls after growth in 2.5 µg/ml 4-NQO for 1.25 hr; in Table 9 the respiratory rate (nmoles/min/mg protein) relative to an untreated control is seen. A comparison of cells exposed to 2.5 µg/ml 4-AQO for 1.25 hr with untreated controls is included. No impairment of respiration appears upon exposure to 4-AQO under these conditions.

The time of onset of drug action was studied by exposing cells to 4-NQO in the polargraph reaction chamber and measuring changes in the rate of O_2 consumption. As shown graphically in Chart 8 O_2 consumption of 4-NQO exposed cells decreased gradually, but not abruptly as seen with classical respiratory inhibitors such as NaN_3 and KCN. The decrease in respiratory rate, expressed as per cent of controls, was 55% at 6-7 min post-drug addition. Under normal conditions the total dissolved O_2 concentration decreased and is accompanied by a decrease in respiratory rate (compare slope of control line at 1-2 min and 5-6 min intervals). In compensating for this natural decrease respiratory rates of control and experimental cells were compared at identical time intervals.

LEGEND CHART 8

Inhibition of Cellular Respiration by 4-NQO. Six ml of a washed cell suspension of ~20 X 10^7 cells/ml in sucrose buffer was added to the reaction chamber and allowed to equilibrate for 1/2 hr. 4-NQO was then added to a final concentration of 14 µg/ml to compensate for the high cell concentration required for polarography. The lines shown were traced from the original recordings.

CHART 8

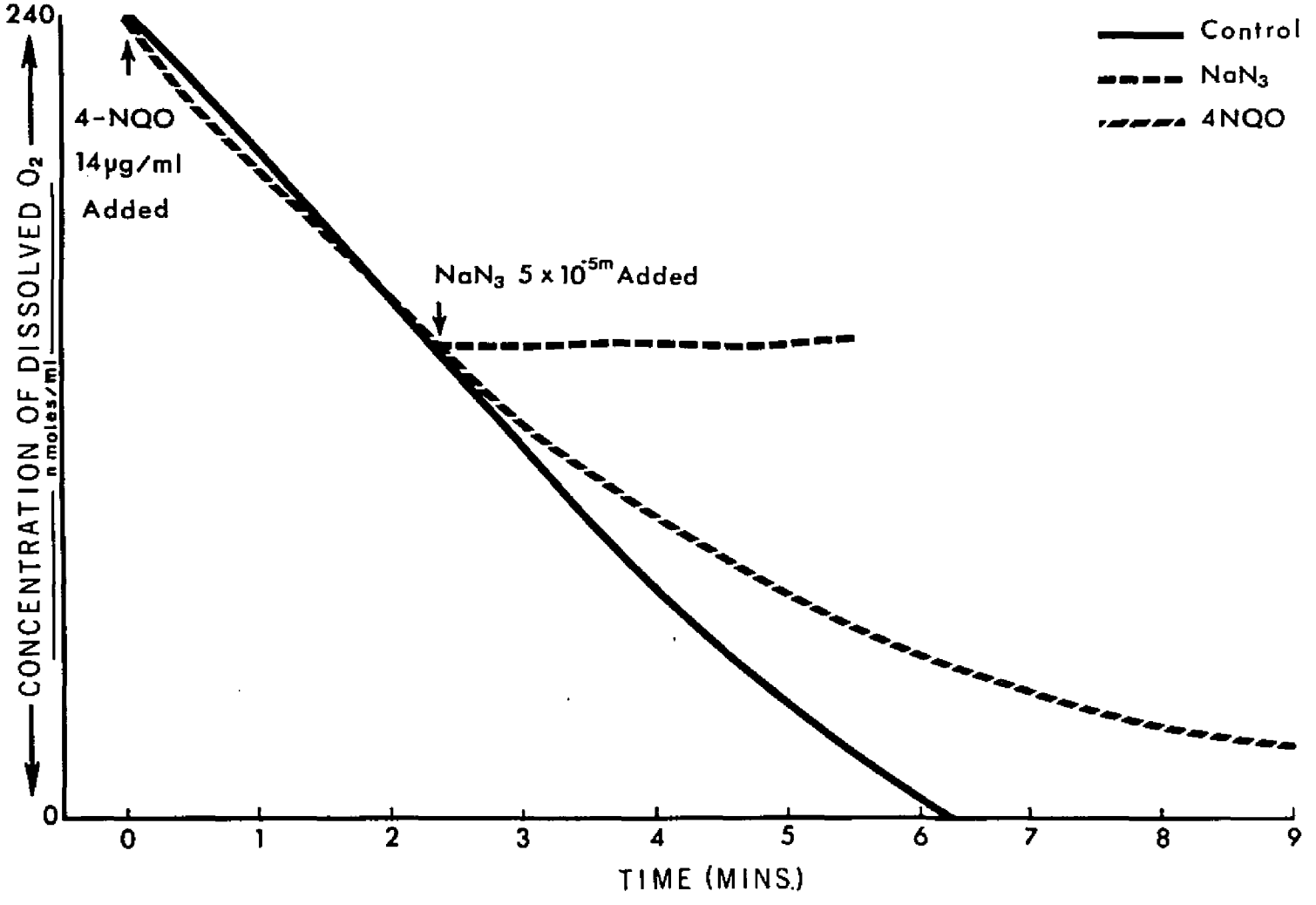

240
0
CONCENTRATION OF DISSOLVED O_2 n moles/ml
4-NQO
14µg/ml
Added
NaN_3 5×10^{-5m} Added
Control
NaN_3
4NQO
0
1
2
3
4
5
6
7
8
9
TIME (MINS.)

TABLE 9

Effect of 4-NQO on Cellular Respiration. Three 1-liter flasks containing 500 ml each were inoculated with 5.0 ml of a log-phase culture of Crithidia. At 33 hr 2.5 µg/ml 4-NQO, 4-AQO respectively were added. After 1.25 hr the cells were prepared for polarography as described in "Methods". The values given represent the average of 3 separate determinations.

	4-NQO	4-AQO	Control
Respiratory rate	19	34.0	32.5
(nmoles/min/mg protein)	17	34	30
	15	34	32
Mean ± (SD)	17 ± 2	34 ± 0	31.5 ± 1
% of control	~50	~100	--

DISCUSSION

The mechanisms which govern 4-NQO transport are poorly understood. We have directed our attention to two aspects of this problem: (a) the role of pH (ionization) and (b) the role played by physico-chemical interactions between 4-NQO and components of the growth medium.

Hutner et al. (1967), in the only reference to the role played by pH in 4-NQO transport, suggested that low pH would favor penetration by impeding dissociation of protons from the 4-nitro group in a manner similar to that shown for 2,4-DNP (pK$_a$ 4.0) (Albert, 1965). (The inability of molecules to permeate natural membranes in their ionized form is discussed by Davson & Danielli (1970).) However, the resemblance between 4-NQO and 2,4-DNP is at best a superficial one. The phenolic hydroxyl of DNP possesses a dissociable proton but there is no equivalent group on the 4-NQO molecule. Due to the strong electron-withdrawing character of the N-oxide group the 4-nitro group is polarized, that is it exhibits a dipole moment, but it does not possess a dissociable proton. Hence the electrostatic character of the molecule is not influenced by fluxes in pH, and in turn pH does

not influence penetrability through natural membranes. This helps to explain our finding that 4-NQO readily penetrates the outer membrane at physiological pH (Chart 1); and also explains why the pKa of 4-NQO is not listed in the standard reference (Ochiai, 1967) and could not be determined in our laboratory by conventional titration techniques.

The second aspect of transport we have studied is the role played by components in the growth medium in facilitating penetration. It is well known that 4-NQO can form charge-transfer complexes with compounds present in the medium such as the aromatic amino acid L-tryptophan (Okano et al., 1969) and nucleobases such as guanine (Nagata et al., 1963). This complex is a relatively weak molecular bond in comparison to covalent, ionic and hydrogen bonds; and consists of the transfer of a negative charge from the highest unoccupied molecular orbital of a donor molecule to the lowest unoccupied orbital of an acceptor molecule. Moreover, Memory (1962) has advanced the notion of a direct association between compounds which can form charge transfer complexes with tryptophan and carcinogenicity. It is conceivable therefore, that 4-NQO is converted by such means into a "transport form" (Korolkovas,

1970) which facilitates penetration.

We approached this question by exposing Crithidia to 4-NQO in distilled H_2O. Our results indicate that 4-NQO readily enters the cell under these conditions (Table 6) from which we conclude that either (a) charge transfer interactions do not occur or (b) that they do occur but play no role in facilitating transport. A not unreasonable result considering the fact that 4-NQO readily induces tumors in mice and rats by simple skin-painting techniques. (Nakahara et al., 1957).

The role played by active transport mechanisms in 4-NQO penetration has been studied by Zahalsky et al. (1963) and Zahalsky & Marcus (1965). These investigators found that inhibition of growth by 4-NQO in O. danica, E. gracilis, C. bovis and the photosynthetic purple bacterium R. palustris was competitively annulled by L-tryptophan (Zahalsky et al., 1963). On the basis of this observation it was suggested that 4-NQO entered the cell by availing itself of a general aromatic transport mechanism, or more specifically by a tryptophan transport system. Accordingly annullment of 4-NQO toxicity was explained by assuming competition between tryptophan and 4-NQO for a common

membrane-bound carrier protein (Singer & Nicolson, 1972).

This hypothesis was tested by the use of a tryptophan-dependent mutant of E. coli which possessed an inducible tryptophan permease (Zahalsky & Marcus, 1965). It was found that prior exposure to non-growth inhibitory doses of 4-NQO resulted in complete inhibition of L-tryptophan uptake within 5 min. Since the converse experiment -- the addition of L-tryptophan followed by measurement of 4-NQO uptake -- was not done, these results are open to two divergent interpretations:

1) 4-NQO saturated the common membrane-bound carrier protein;

2) 4-NQO "dissolved" through the lipoidal layer of the membrane, entered the nucleus, and blocked the operon responsible for regulating synthesis of the inducible tryptophan permease (Boezi & De Moss, 1961).

The latter interpretation is consonant with our findings that 4-NQO acts with extreme rapidity to block RNA transcription in the nucleus. The annulling experiments, on the other hand, point to an active transport mechanism mediated by an aromatic amino acid transport system. At present, then, insufficient experimental evidence is

available to propose either an energy-dependent or energy-independent mechanism of 4-NQO transport.

In summary, it is most unlikely that 4-NQO is converted into a "transport form"; it probably enters the cell in a polarized but electrically neutral state and though evidence has been presented for mediation by a tryptophan permease system, definitive proof is absent.

In order to determine whether the cytotoxic effect of 4-NQO was dependent on the physiological state of the cell population, we exposed Crithidia to 4-NQO at lag, log and plateau phase. These phases are characterized by wide metabolic differences. During lag phase, for example, though biosynthesis occurs, cell division and DNA synthesis are repressed; log-phase growth, on the other hand, is typified by maximal biosynthesis and cell division; at plateau phase, a steady state exists during which the rate of cell division equals the rate of cell death.

Our results show clearly that cytotoxicity is independent of the physiological state of the cell population (Chart 4; Table 7). Kuroki et al. (1970) reported similar results in cultured mammalian cells; in SV-40 infected tissue culture cells expression of the

t-antigen is also independent of DNA replication or cell division (Todaro & Green, 1966) -- a finding which suggests that the early cytotoxic effects of 4-NQO may be similar in nature to t-antigen formation induced by tumor-forming viruses.

Chart 2 shows dose-response growth patterns of Crithidia exposed to increasing concentrations of 4-NQO. Clearly, the degree of inhibition -- measured in terms of the time required for resumption of normal cell division -- is a direct function of the concentration of 4-NQO in the medium. Two conclusions are immediately evident: (a) that the duration of mitotic delay is dose-dependent and not based on an all-or-nothing principle, and (b) that the cytotoxic effect of 4-NQO is reversible within the concentration limits imposed. Later we will discuss the probable role of dark-repair mechanisms in the latter process.

By converting our data by the method of Schubert (1970) into a linear plot of net doubling time vs. 4-NQO concentration, some further conclusions can be drawn (Chart 3). Within the range of 0→1 μg/ml 4-NQO there is a logarithmic increase in the initial doubling time (T_1). An inflection point then occurs following which

concentration increments have a diminishing effect. A curve connecting the experimental points suggests a hyperbolic saturation curve. This This result becomes understandable if we assume that there are a finite number of 4-NQO targets in the cell which determine the cytotoxic effect; when all the "receptor sites" are occupied saturation is reached and no further cytotoxic effect is elicited regardless of the number of new 4-NQO molecules added. The receptor concept as advanced by Ehrlich and later Krebs, is discussed in detail by Albert (1965) and Korolkovas (1970).

The micrographs shown in Figs. 3, 5, 6, 7, 9 represent the first unambiguous demonstration of nucleolar segregation induced by a chemical in a protozoan. The absence of effects on the kinetoplast were surprising but the induction of nucleolar alterations was not.

Since the original report by Endo et al. (1959) of the induction by 4-NQO and its carcinogenic derivatives of morphological alterations in the nuclei of cultured mammalian cells, seven papers have appeared in the literature dealing with different aspects of this phenomena (Endo et al., 1961a; Harada et al., 1968; Hayashi & Hasegawa, 1970; Lazarus et al., 1966; Mita et al., 1965; Paul et al., 1971b; Reynolds

et al., 1963).

Endo et al. (1959) observed by light microscopy and histochemical techniques that mouse kidney or Chang liver cells exposed to 10^{-5} M 4-NQO exhibited nuclear inclusion bodies characterized by Feulgen and PAS-negative and pyronine-positive reactions. Endo et al. (1961) later showed these inclusion bodies to be RNP in nature. Nuclear inclusions were induced only by carcinogenic derivatives of 4-NQO indicating that these cytological changes were related in some way to the carcinogenicity of 4-NQO.

With the advent of the electron microscope more precise examination of the cytological effects of 4-NQO became possible. Reynolds et al. (1963) was the first to describe the fine structural changes induced by 4-NQO. Using time-lapse cinematography and electron microscopy the following changes in Chang liver cells exposed to 10^{-5} M 4-NQO for 10 min were observed:

(a) a progressive decrease in nucleolar size;

(b) redistribution of nucleolar components into light and dark "nucleolar caps";

(c) appearance of nuclear inclusion bodies.

Similar observations were later made in cultured rat nephroblastoma cells, Chang liver and HeLa cells (Lazarus et al., 1966) and in TTC-13 cells derived from mouse ascites (Harada et al., 1968). In the latter multivesicular bodies appeared in the cytoplasm along with nucleolar changes.

Recently, Paul et al. (1971b) have shown that weakly carcinogenic derivatives of 4-NQO induced correspondingly feeble nucleolar changes, suggesting that the intensity of carcinogenicity was quantitatively related to the degree of nucleolar alterations observed. 4-NQO induced nucleolar alterations have also been demonstrated in vivo by Hayashi & Hashigawa (1970) in rat lung tissue. Their finding is of particular interest in that 4-NQO is a potent inducer of pulmonary tumors in rats; again suggesting that induction of nucleolar changes is causally related to malignant transformation.

The only reported study of the effect of 4-NQO on nuclear structures in protozoa were on the ciliate Tetrahymena pyriformis. Synchronously grown Tetrahymena exposed to 20 µg/ml 4-NQO exhibited

the following changes (Mita et al., 1965):

(a) distorted karyokinesis;

(b) uneven distribution of DNA to daughter cells;

(c) irregularities in daughter cell size.

Ultrastructural changes included nucleolar alterations analogous to those described in Crithidia but the small size of the interphase nucleoli of Tetrahymena precluded a convincing description (Endo et al., 1971). Again, as in previous reports, these changes were induced only by carcinogenic derivatives of 4-NQO.

To summarize: our results are in close agreement with those of previous workers. Nucleolar alterations were induced at similar dosages, and after similar periods of exposure. Also, inclusion bodies, probably similar to those originally described by Endo et al. (1959) were found associated with nucleolar changes. In addition, these changes were induced only by carcinogenic derivatives of 4-NQO.

At the time of Reynold's report the SH substitution reaction was considered by some to be the key to the carcinogenicity of 4-NQO. (For a review of the role of SH groups in carcinogenesis see Harington

(1961).) Reynolds based his explanation of "nucleolar caps" on the finding of Fukuoka et al.[1] (1959) that 4-NQO inhibited glycolysis in Ehrlich ascites cells presumably by inactivating the SH-containing enzyme, glyceraldehyde-3-phosphate dehydrogenase. A reconstruction of the proposed sequence of events is as follows: exposure to 4-NQO resulted in the inhibition of glycolysis which in turn resulted in a decrease in cellular NADH. This depressed ATP levels and ultimately inhibited protein and nucleic acid synthesis. In response to the depression in protein synthesis the cell was envisaged to discharge pre-formed RNP from the nucleus to the cytoplasm in an attempt to restore normal rates of protein synthesis.

Reynolds admirably included most of the known facts available at the time concerning the biochemical effects of 4-NQO. However he failed to take into account Tomaru et al.'s (1961) finding that although 4-NQO had a stronger affinity for SH groups than the related compound, 4-nitroquinaldine N-oxide, the latter had a more marked effect on glycolysis than the former. Hence the connection between SH substitution and inhibition of glycolysis upon which Reynolds

based his hypothesis was largely unsubstantiated.

Later, Lazarus et al. (1966) modified Reynolds' hypothesis on the basis of the finding that Act D induced nucleolar segregation in mammalian cells. They speculated on the role of 4-NQO in blocking RNA synthesis in the nucleus, but could not formulate an hypothesis because binding to DNA by 4-NQO was yet to be demonstrated.

On the basis of current knowledge of the nucleolus and agents which affect its morphology, it is now clear that 4-NQO acts by physically binding to nuDNA (Busch & Smetana, 1970). The nucleolus is presently the recognized site of synthesis and processing of rRNA (Brown & Gurdon, 1964; Darnell, 1968; Perry, 1969) and contains in its associated DNA cistrons coding for rRNA (Ritossa & Speigelman, 1965). In addition, the metabolism of rRNA in the nucleolus has been clarified after several years of intensive study (Darnell, 1968): the first rRNA product transcribed is a long polynucleotide chain which sediments in sucrose gradients as a 80-110S RNP particle and contains 45S RNA. This long polymer is then cleaved in a non-conservative process (Willems et al., 1968) into 28S and 18S ribosomal sub-units

which are also found in the nucleolus. In Crithidia the sequence is similar except that a large 35S RNA is initially synthesized and subsequently cleaved into 18S and 28S RNA (Gottlieb & Zahalsky, 1968).

The processing sequence is correlated with morphological elements in the nucleolus in the following manner:

DNA $\overset{(1)}{\rightarrow}$ [Fibrillar component] $\underset{(3)}{\overset{(2)}{\rightleftharpoons}}$ [Granular component] $\overset{(4)}{\rightarrow}$ Cytoplasm

[Adapted from Simard (1970)]

Evidence in support of this sequence has been reviewed (Busch & Smetana, 1970; Simard, 1970) and particularly convincing electron micrographs (Miller et al., 1971) and combined autoradiographic and biochemical evidence has been presented by Das et al. (1970); Granboulan & Granboulan (1965); Jacob (1967); Liau & Perry (1969); Samashima et al. (1970) and Unuma et al. (1968). It is generally agreed therefore that inducing agents, like 4-NQO, act by blocking reaction 1: the transcription of ribosomal DNA cistrons into 45S RNA (Busch & Smetana, 1970; Simard, 1970). Accordingly, the molecular events which correspond to our morphological observations are reconstructed as follows:

Upon exposure to 4-NQO, nucleolar DNA is altered in such a manner

that reaction 1 is blocked, and synthesis of 45S RNA shut down (Chart 5). Since no new 45S RNA is synthesized, only that pre-existing remains; as a result it is slowly depleted by combined reactions 2, 3, and 4 which serve to convert 45S into ribosomal sub-units and transport them to the cytoplasm despite the reverse effect of reaction 3 (Figs. 5, 6, 7, 9). This is seen as a gradual depletion of the granular elements until only a small fragment -- a fibrillar remnant -- remains of the original nucleolar mass (Fig. 9).

Explanation of the topological changes which result in the segregation of granular and fibrillar elements is based largely on analogies with lampbrush chromosomes and chromosome puffs (Busch & Smetana, 1970). The loops and puffs consist primarily of dispersed, actively synthesizing DNA in contrast to the rest of the chromosomal DNA which exists in a contracted inactive form; when exposed to Act D the loops and puffs immediately retract and RNA synthesis stops (Ebstein, 1969; Snow & Callan, 1969; Stevens, 1964). A similar dispersed DNA is thought by some (Barr & Plaut, 1966; Unuma et al., 1968) to serve as the structural framework of the nucleolus. Consequently, interaction

between an inducing agent and the dispersed DNA is envisaged to result in the collapse of nucleolar architecture i.e. nucleolar segregation. Variations on this theme have been presented by Goldblatt & Sullivan (1970) and Recher et al. (1971).

The inhibitory effects of 4-NQO on RNA biosynthesis reported here are in agreement with the earlier findings of Paul in isolated Chang liver nuclei (1967), intact Chang liver cells (1966) and HeLa cells (1969); which in turn are in agreement with Simards' (1970) generalization that all agents which induce nucleolar segregation simultaneously block RNA transcription. Paul et al. (1969) found, as we did, that labeled uridine (uracil) was incorporated linearly over a period of 1 hr, and that upon exposure to 4-NQO uptake was immediately blocked. He also made the additional observation that the decrease in macromolecular RNA synthesis was associated with a parallel increase in acid soluble RNA. Similar findings were reported by Amsterdam et al. (1967) using PCA extraction techniques

The relation between the decrease in TCA-insoluble and increase in TCA-soluble RNA was clarified by Tada et al. (1970) by measuring

changes in the template ability of liver cell DNA isolated from HAQO-treated rats in an E. coli RNA polymerase system. The technique used allowed for simultaneous determination of the number of chain initiation points and the length of the RNA's synthesized. It was found that DNA altered by HAQO synthesized numerous short RNA's by increasing the number of chain initiation points. Thus the block in RNA synthesis is now seen as the net effect of converting the transcription process from one synthesizing small numbers of long chains to one synthesizing large numbers of small chains (Diagram 4).

The major objection to this experiment is that an E. coli RNA synthesizing system was used rather than a eukaryotic system; therefore one cannot readily extrapolate Tada's results to eukaryotes. Recent reports concerning the isolation of multiple RNA polymerases from eukaryotic cells may provide a means to overcome this objection (Horgan & Griffin, 1971; Roeder & Rutter, 1970; Tocchini-Valentini & Crippa, 1970). Isolation of RNA polymerases which transcribe different classes of RNA in vitro could provide an opportunity to test transcription of 4HAQO (4-NQO)-altered DNA in a complete eukaryotic system.

LEGEND DIAGRAM 4

Schematic diagram of the effect of 4-NQO on RNA synthesis, illustrating the induction of increased chain initiation points during RNA transcription. Note that each newly transcribed chain has only one γ-phosphate at its initiation point.

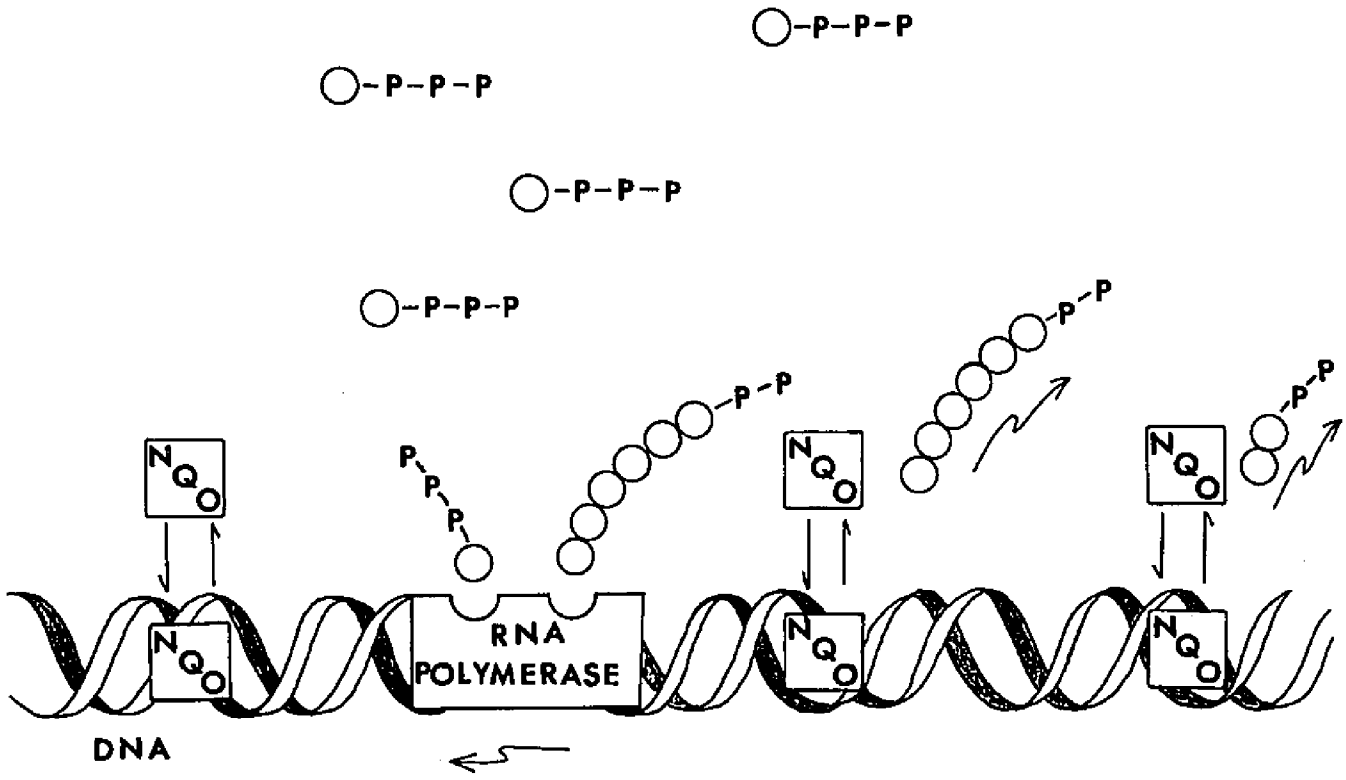


DIAGRAM 4

Further insight into the effect of 4-NQO on RNA synthesis was gained by Floyd et al. (1968). They compared changes in ^{32}P-labeled whole-cell RNA after exposure to 4-NQO, aflatoxin B, and Act D; exposure to either carcinogen did not alter whole cell RNA nucleotide composition. In contrast, Act D preferentially blocked synthesis of GC-rich 45S precursor, thus making RNA synthesized in the presence of Act D higher in AMP and UMP than in untreated cells. These findings suggest that the carcinogens, aflatoxin, and 4-NQO, unlike Act D, act randomly along the template rather than by specifically blocking cistrons coding for 45S RNA.

The inhibition of respiration by 4-NQO reported here is in agreement with the results of Hutner et al. (1967) for Euglena gracilis. In Chart 8 it can be seen that, in comparison with azide, 4-NQO slows but does not sharply inhibit, the respiratory rate of Crithidia which suggests that 4-NQO acts at a distance from the terminal oxidase. We investigated this possibility by inspecting difference spectra in the presence and absence of 4-NQO; 4-NQO-treated preparations were indistinguishable from untreated preparations. Such a result suggests,

but does not prove, that 4-NQO acts at a site other than the cytochrome system. These findings may serve to distinguish 4-NQO from the related compound 2-n-heptyl-4-hydroxyquinoline N-oxide which acts as an uncoupling agent at the antimycin-sensitive site in mitochondria (Lightbown & Jackson, 1956) and chloroplasts (Izawa et al., 1966). There are no reports on morphological changes induced by 2-n-heptyl-4-hydroxy-quinoline N-oxide in nuclei or other cell structures.

The latency of the inhibitory curve (Chart 8) and the partial nature of the respiratory blocks induced by 4-NQO (Table 10) may be explained on the basis of the chemi-osmotic hypothesis (Mitchell, 1966). According to Mithcell, the lipid coat of the mitochondria serves to insulate the respiratory chain from the aqueous phase. Since 4-NQO is soluble in both aqueous and organic solvents, it might alter the architecture of the lipid coat, thereby inducing an electron leak. As a result: (a) though dehydrogenation may proceed at a normal rate at the substrate end of the respiratory chain, a fraction of the electrons would not reach the terminal electron acceptor, oxygen. Then, (b) the respiratory rate would (1) gradually diminish (2) be inhibited only partly (Chart 8 and Chart 10).

Lastly, one cannot exclude the possibility that inhibition of respiration is due to gross damage to nDNA or to sub-threshold damage to kDNA. Since the structure and continued functioning of the mitochondrion (and chloroplast as well) is controlled by a dual regulatory system involving interactions between nuclear DNA and organellar DNA, lesions in either class of macromolecule could be responsible for the latency and imcompletness of the respiratory inhibition (Attardi & Attardi, 1970; Kroon, 1969; Preer, 1971, among others). Similar reasoning can be applied to explain the arrest in chloroplast development reported by Hutner et al. (1967) following exposure of Euglena gracilis to 4-NQO. The definitive experiment to determine the role of kDNA in the 4-NQO effect depends on the isolation of intact mitochondria showing coupling and respiratory control from Crithidia; a technical problem which has not yet been solved (Hill & Anderson, 1970).

The cloning results generally resemble those obtained in mammalian cells (Horikawa, et al., 1969], bacteria (Bond et al., 1970; Okabayashi et al., 1965], yeast (Nagai, 1969), and phytoflagellates (Hutner, et

al., 1967), the main difference being the extreme rapidity with which inhibition takes place in Crithidia (Chart 7).

Since clone formation is the end result of a chain of events which includes DNA replication and distribution, cytokinesis and membrane-substrate interactions, damage at any one of these processes could impair clone formation. In Crithidia exposed to 4-NQO, an occasionally large cell appears having two flagella, two kinetoplasts, and a single enlarged nucleus. Apparently nuclear and kinetoplast genomes had replicated but cytokinesis was arrested. A similar post-replicative effect has been reported in Tetrahymena; by Mita et al. (1969) and Kuroki et al. (1970) in dividing mammalian cells exposed to 4-NQO.

Impairment of clone formation could also be due to inactivation by the SH substitution reaction of SH-containing proteins essential for normal mitosis as shown in Sea Urchin eggs by Kamahora & Kakunaga (1968).

It is unlikely on kinetic grounds that inhibition of the energy-generating system explains the cloning results. Comparison of Charts 7 and 8 shows clearly that a major reduction in clone-forming ability occurs before significant decrease in respiratory rate.

Morphological and biochemical alterations induced by AF and 4-NQO are compared in Table 10. Clearly the biochemical changes induced by these agents reflect selective damage to the kinetoplast and nucleus respectively. What physico-chemical differences between nDNA and kDNA might determine such selectivity?

First: kDNA and nDNA are topologically distinct molecules. kDNA, like viral, bacterial, and mDNA consists largely of covalently closed circular molecules (Borst & Kroon, 1969; Helinski & Clewell, 1971), whereas nDNA is linear. kDNA differs from other circular DNA's by virtue of its small size; the average contour length of kDNA is 0.45 μ whereas that of mDNA is 5.0 μ (Laurent & Steinert, 1970; Renger & Wolstenholme, 1971; Riou & Delain, 1969; Riou & Pautrizel, 1969; Simpson, 1972; Simpson & DaSilva, 1971). Selective binding may therefore be determined by the different geometric properties of kDNA and nDNA. Supporting this notion is that AF interacts selectively with other closed circular cytoplasmic DNA's such as the R factor in Enterbacteriaceae (Anderson, 1968) and the transmissible F factor in E. Coli (Valentine et al., 1969). Future determination of the role played by circularity/linearity, by applying in vitro binding techniques,

may serve to clarify this point.

Second: nuclear and kinetoplastic DNA's differ in that chromosomes exist as nucleoprotein complexes in sharp contrast to prokaryotic DNA, mDNA, and kDNA (Borst & Kroon, 1969; Delange & Smith, 1971; Nass, 1969; Steinert, 1965; Stellwagon & Cole, 1969; Swift & Wolstenholme) which exist as naked DNA molecules. Conceivably, masking of negatively charged surfaces by associated proteins may serve as a selective barrier to the positively charged AF molecule. Since AF is also larger than 4-NQO, steric interactions may serve to hinder access to DNA.

Third: in addition to the absence of associated proteins, kDNA differs from nDNA by virtue of a higher AT/GC base ratio (Renger & Wolstenholme, 1970; Riou & Pautrizel, 1969). The relatively higher GC content of nDNA could bias sensitivity towards 4-NQO since the 4-NQO molecule presumably binds preferentially to deoxyguanoside residues (Okano & Uekama, 1967; Paul et al., 1971) whereas AF intercalates between base pairs (Waring, 1968).

TABLE 10

Drug	Site of Action	Ultrastructural Changes	Biochemical Changes	Physiological Changes
4-NQO 10^{-6} M	Nucleus (Nucleolus)	95% nucleolar segregation after 1.25 hr	(1) rapid selective inhibition of of RNA polymerase; 100% by 5 min. (2) decrease in 45S RNA precursor; increase in 10S RNA (Lazarus et al., 1966; Paul et al., 1969; Tada et al., 1970).	(1) cells from all stages equally susceptible. (2) respiration impaired 50% at 1.25 hr.
Acriflavin 10^{-6} M	Cytoplasm (Kinetoplast)	(1) 90% dyskinetoplasty after 3 days (Simpson, 1968a; Steinert et al., 1969). (2) 80% after 24 hr in Crithidia (Hill & Anderson, 1969).	(1) rapid selective inhibition of kDNA synthesis 50% by 20 hr (Hill & Anderson, 1969). (2) (a) satellite band of kDNA disappears (Simpson, 1968a; Steinert & van Assel, 1967). (b) circular molecules disappear (Renger & Wolstenholm, 1971). (3) cytochromes $\underline{a_1}$ $\underline{a_3}$ $\underline{c_{555}}$ and $\underline{b}$ decreased (Hill & Anderson, 1970) also SDH, L-GPDH. (4) glycolytic enzymes increased esp. G-6-P-D (Bacchi & Hill, 1972).	(1) resting cells from stationary phase more resistant to the drug than actively dividing cells (Simpson, 1968a). (2) respiration impaired (a) in L. tarendolae 10% in 20 hr, 90% in 70 hr (Simpson, 1968a). (b) in C. fasc. 50% (Hill & Anderson, 1969; Bacchi & Hill, 1972). (3) cloning impaired 100% by 4.5 divisions (Simpson, 1968a).

Finally, differences in ionic milieu and membrane permeabilities may also influence binding selectivity. AF, because of its larger size and positive charge (Albert, 1965) may not be able to penetrate the nuclear membrane; on the other hand, it may easily penetrate the nucleus but be neutralized by negative ions present in the nuclear sap. In either case binding to nDNA would be prevented.

Apparently a priority governs binding of 4-NQO: under our experimental conditions nDNA is selectively altered and kDNA is not. However, as noted in Table 10 dyskinetoplasty is usually observed after 2-3 days exposure to AF whereas nucleolar segregation was induced in Crithidia by exposure to 4-NQO for only 1.25 hr; exposure to 4-NQO for longer periods may serve to ascertain what long-term kinetoplastic effects, if any, may occur.

In addition, our observations do not rule out binding to cellular DNA's present in quantities below the threshold of visualization. One such species may be the recently isolated closed circular DNA presumably from yeast peroxisomes (Clark-Walker, 1972).

Genetic analysis of yeast petites induced by 4-NQO strongly

suggests sub-threshold binding to mDNA; yeast petites induced by 4-NQO are largely cytoplasmic, i.e., do not exhibit Mendelian patterns of inheritance, not karyotic, as might be expected from our results (Nagai, 1969a). This was detected by the use of a diploid yeast strain which will not sporulate if a double recessive nuclear gene mutation occurs. Since RD mutations are always recessive if most cells in the final population of a treated diploid are viable, and a fraction of these are RD, they are considered to be cytoplasmic in origin. However, definitive proof requires crosses between haploid strains followed by tetrad analysis and these have not been reported at present. Further investigation has revealed that a wide spectrum of mutants ranging from cytoplasmic to partial karyotic to full karyotic petites can be induced by 4-NQO (Morita & Mifuchi, 1965; Nagai, 1969b; 1969c). The mutation induced by 4-NQO was unusual in that intermediate mutants were observed in addition to the typical RD mutant; when the TTC overlaying method was used for the detection of RD colonies of yeast treated with AF only red (normal) and white (RD) were found, while those treated with 4-NQO produced a wide range of colonies ranging from pink, sectored,

and deep red (respiratory ultra-sufficient) to normal RD. This variety of colonies resembled very closely those of UV irradiated yeasts and underscores the radiomimetic activity of 4-NQO. The higher sensitivity of genetic analysis indicates that overt nuclear changes such as we have found in Crithidia may be too crude an index of selective binding by 4-NQO.

Lastly, we do not know the effect of changes in nutritional conditions. In our experiments Crithidia was grown in two basically different media, defined and crude; perhaps if a radically different medium was used Crithidia would respond in an altogether different manner to 4-NQO.

The early events following exposure to 4-NQO have been described; their relation to earlier experiments discussed; and some inferences drawn concerning the selectivity of 4-NQO binding. We may now consider what relation, if any, these changes have to malignant transformation. Three possibilities can be considered. The early changes could be either (a) necessary and sufficient; (b) necessary but not sufficient; of (c) neither necessary nor sufficient for malignant transformation.

Arguments for each of these possibilities follow.

If we consider the early changes induced by 4-NQO to be aspects of a general reversible cytotoxic effect then condition (c) might prevail. Reversibility of cytotoxicity implicit in the recovery from mitotic delay shown in Chart 2.

The cytotoxic effect of carcinogens has long been known and was formally linked to tumor formation by Haddow (1938a). However, recent evidence (DiPaolo, 1971) suggests, to the contrary, that transformation can take place independently of cytotoxic effects. If DiPaolo's work is corroborated in other systems, then the early changes we have reported may prove to have no bearing whatsoever on later malignant changes.

The argument in favor of a necessary but not sufficient condition is simple: over 30 chemical, physical and infectious agents, carcinogenic and non-carcinogenic, are currently known to induce nucleolar segregation in mammalian cells in culture and plant root tips (Busch & Smetana, 1970). These include aflatoxin B_1 (Bernhard et al., 1965; Edwards et al., 1971; Pong & Wogan, 1970), Act D (Reynolds et al., 1964; Schoefl, 1964), mitomycin C_1 (Lapis & Bernhard, 1965), cordycepin

(Stockert et al., 1970), UV irradiation (Montgomery & Reynolds, 1965) and herpes simplex virus (Sintori & Bassisio-Bestetti, 1967). Recently Stockert (1971) has shown that cold, 2.4-DNP and anoxia also induce nucleolar alterations. Since such a diversity of compounds elicit the same morphological effect, nucleolar segregation cannot possible serve as a necessary and sufficient condition for carcinogenesis. On the other hand, since AQO and other non-carcinogenic derivaties of 4-NQO fail to induce such changes one may be forced to conclude that nucleolar segregation is a necessary but insufficient condition for malignant transformation.

Evidence in support of a necessary and sufficient argument center around the work of Duryee (1969) and Harris (1970). Duryee found in amphibian adenosarcoma, induced by lead acetate, that nucleolar rearrangements invariably preceded transformation. He thereupon hypothesized that the nucleolus played a "pacemaker" role in the normal cell -- one primarily concerned with regulating the rate of cell division. According to his hypothesis, interaction between a chemical carcinogen and the nucleolar "pacemaker" resulted in a re-setting of

the controls for cell division, i.e., transformation. Harris arrived at a similar "pacemaker" function for the nucleolus from a different experimental viewpoint. By the use of heterokaryons Harris found that transfer of informational RNA to the cytoplasm was obligatorily coupled to normal nucleolar function. Inactivation of the nucleolus invariably resulted in cessation of the flow of all species of RNA from nucleus to cytoplasm; hence the conclusion that the nucleolus served a regulatory function by controlling the flow of information from nucleus to cytoplasm. Although Harris' views have been sharply criticized especially by Gurdon & Ford (1966) and Perry et al. (1970), they have served to stimulate research into nucleolar function.

Nucleolar changes preceding cancerization similar to those described by Duryee have been reported by Yoshida et al. (1970) and Isaka (1970) in mammalian cells exposed to 4-NQO *in vitro* and by Hayashi & Hasegawa (1970) in rat alveolar cells treated *in vivo* with 4-NQO. These considerations lead to the idea that the nucleolus may serve a function other than the two usually ascribed to it, i.e., (1) site of the NOR (McKlintock, 1934) and (2) site or rRNA synthesis and processing

(Darnell, 1969); a third site (3) is conceived to serve a regulatory function and to be susceptible to chemical carcinogens.

The use of new techniques such as the argon laser (Berns et al., 1971) to be selectively inactivate minute regions of the nucleolus should provide more information on the nature of the hypothetical nucleolar "pacemaker".

4-NQO induces single and double strand scissions in DNA *in vitro* (Sugimura, 1968) and *in vivo* (Andoh & Ide, 1972; Horikawa et al., 1970; 1972; Tada et al., 1970) and the latter scissions are restituted by "unscheduled" DNA synthesis in the dark. Moreover, UV- and NQO-damaged DNA is repaired by the same photoreactivation mechanism in bacteria (Kondo & Kato, 1968). These phenomena comprise what are known as the "radiomimetic" properties of 4-NQO. Alexander (1969) has discussed the various, often misleading, connotations of the term "radiomimetic".

The steps in the dark repair process have been clarified recently in bacteria and eukaryotic cells (Dalrymple et al., 1970; DuPraw, 1970; Setlow & Carrier, 1964). In outline, dark repair consists of four steps: (1) damaged bases are recognized and single-strand scissions make in

the DNA helix by a specific endonuclease; (2) an exonuclease excises a number of bases along the strand; (3) the excised region is restored by unscheduled DNA synthesis; and (4) the physical continuity of the strand is restored by a DNA ligase.

Horikawa et al., Tada et al., and Andoh & Ide therefore have indirectly demonstrated step 1 and step 3 of the process. Proof for step 2 is still wanting and requires demonstration of the elimination of 4-NQO-modified bases into the medium in a way similar to that described by Roberts et al. (1968) for ^{35}S-containing sulfur mustards. The ligase reaction is assumed to occur during 4-NQO repair but it too has not been definitively shown.

In all likelihood 4-NQO does not cause direct in vivo scissions in the DNA helix; instead it probably binds to and distorts bases or the ribose-phosphate backbone in such fashion as to induce dark-repair mechanisms (Dalrymple et al., 1970). Hence, in vivo scissions induced by 4-NQO are of an entirely different nature than those induced in vitro.

Dark repair process may be responsible for the delay in and subsequent resumption of growth depicted in Chart 2. A similar delay

and resumption was reported by Andoh & Ide (1972) during repair synthesis in mammalian cells exposed to 4-NQO.

Since the repair process presumably evolved as a device to maintain the integrity of the genetic material it is a priori a prime target for carcinogens which induce defects in DNA. In addition, defective repair may permit permanent changes in DNA which lead ultimately to cancer.

The only report extant showing a direct causal relationship between defective repair processes and cancer is that by Cleaver (1968) on skin cells from patients with the rare malignant disease Xeroderm pigmentosum. Individuals with XP are highly prone to develop skin cancers from exposure to sunlight. Cleaver found that fibroblasts from XP patients could not repair UV-induced lesions in their DNA. As shown later, XP cells lacked the endonuclease (Setlow et al., 1969); and more recently Stich & San (1971) have shown that XP cells were unable to repair 4-NQO-induced damage to DNA.

Although no other reports of a similar nature have been forthcoming, Cleaver's work has served to stimulate further interest in repair

processes and cancer. Epstein et al. (1971) has reviewed some recent developments in this area.

Repair processes may underlie several hitherto unexplained phenomena involving 4-NQO, such as production of chromosomal abnormalities, i.e., breaks and bridges, in mammalian cells (Kihlman, 1971; Stich & San, 1970; Yoshida, 1970) and induction of phage by 4-NQO (Calendar, 1970; Endo et al., 1963a). Moreover, cell lines with repair systems characterized by different specificities could account for the variable sensitivity of different cell lines to 4-NQO (Amsterdam et al., 1967; Horikawa et al., 1969). Repair mechanisms may also be implicated in the heretofore unexplainable drop in NAD concentration following exposure to 4-NQO reported by Ono et al. (1959). It has recently been shown that AMP derived from NAD serves as an obligatory cofactor in the ligase reaction (DuPraw, 1970; Gumport & Lehman, 1971). In this reaction DNA-ligase combines with NAD yielding an enzyme-adenylate complex and free nicotinamide; the ligase-adenylate complex then joins single-strand breaks and adenylic acid is released. Hence the drop in NAD after exposure to 4-NQO may be explained by assuming the induction

of repair enzymes which in turn cause the breakdown of NAD to allow the continued functioning of the ligase reaction. This does not exclude the possibility that the NAD decrease is caused by destruction of NAD synthetase which is located in the nucleolus (Busch & Smetana, 1970) followed by exhaustion of the pre-formed NAD pool.

Repair processes may also serve to explain the three hypotheses discussed earlier. For example, if nucleolar changes are irrelevant to transformation one can postulate damage to extra-nucleolar DNA followed by mis-repair and resulting in malignant transformation whether by oncogene, mutation, or activation of a latent virus. On the other hand, if nucleolar changes are a necessary but <u>not</u> sufficient condition, mis-repair could occur in both nucleolar or non-nucleolar regions involving the "pacemaker" region and non-nucleolar DNA. In the last case, abnormal repair processes induced by 4-NQO in the nucleolus alone could result in permanent alteration of a hypothetical nucleolar "pacemaker".

Obviously, detailed understanding of the repair mechanism is required before these hypotheses can be put to decisive test.

As mentioned in the "Introduction", it is generally believed that

4-NQO is converted enzymatically to 4HAQO and then to a variety of intermediates including the diacetyl derivative (Enomoto et al., 1968) and a free-radical intermediate (Nagata et al., 1966a; Nobuko et al., 1968; Okano & Mekama, 1968; Okano et al., 1970). In this sense 4-NQO differs from typical alkylating agents such as sulfur mustards which spontaneously form a highly reactive cationic intermediate which binds in a one-step process to DNA (Miller & Miller, 1969); and has much in common with the aromatic amines typified by acetylaminofluorene (Miller, 1970) which are characterized by a two-step reaction involving an hydroxylated intermediate.

One argument for NQO being converted into an electrophilic (electron-deficient) reactant is that toxicity is opposed by cysteine, tyrosine, tryptophan which as Miller & Miller (1969) pointed out are likely targets of electrophils. The most convincing evidence that HAQO is the proximate carcinogen and 4-NQO the pre-carcinogen is that 4-NQO is biologically inert in systems devoid of enzyme reducing the nitro group. Thus, inactivation of phage T_4 (Ishizawa & Endo, 1967; Yamamoto et al., 1970) and B. subtilis transforming DNA (Tada et al., 1967) occurs only after exposure to 4HAQO and not 4-NQO. Moreover,

4HAQO, but not 4-NQO induces single-strand scissions in isolated DNA (Sugimura et al., 1968).

However, several observations remain to be accounted for before this hypothesis can be accepted. For example, in repair experiments, higher concentrations of 4-HAQO than of 4-NQO were required to cause single-strand scissions *in vivo* (Honikawa, 1970). Moreover, Endo succeeded in inducing mutants in tobacco mosaic virus by exposure to 4-NQO *in vitro* (Endo et al., 1961) -- a system totally lacking in reducing enzymes. In *Tetrahymena*, 4HAQO was found to be 1/5th as effective as 4-NQO in causing aberrant cell division (Mita et al., 1969). And lastly, Hückel molecular orbital computations revealed that at least theoretically either 4-NQO or 4HAQO could interact with deoxyguanosine residues (Paul et al., 1971).

Our results highlight some kinetic aspects of this problem: the extreme rapidity of 4-NQO action implies either that metabolic reduction does not take place, or that metabolic reductions must occur nearly instantaneously. Further study of the kinetics of these reactions would be helpful in accounting for the results presented here.

RESULTS

Part II

a) Selection Procedure

When Crithidia was cloned on an agar containing 4-NQO at concentration of 0.2-0.3 µg/ml, a few small colonies could be seen arranged like a strand of beads of gradually diminishing diameters at the perimeters of the plate. Concentrations above 0.5 µg/ml completely inhibited clone formation (Table 11). Since we sought clones which were metabolically altered but, of course not all killed, plates containing 0.25 µg/ml 4-NQO were used to select clones with deficient respiratory metabolism as evidenced by inability to reduce tetrazolium.

Selection proceeded as follows: agar plates containing 4-NQO were inoculated with various concentrations of Crithidia cells and incubated at 27 C for 9-12 days. After colonies had developed, the plates were overlaid with TTC agar; 2-3 days elapsed before examining plates for white colonies. The latter were picked and inoculated into liquid medium; when the inoculum had grown to a sufficient density the sequence of steps were repeated. The process was repeated for 9 consecutive selections.

TABLE 11

Colony formation by Crithidia on agar containing 4-NQO. The stock solution of 4-NQO was 100 µg/ml in distilled water. It was diluted with water as needed. Appropriate amounts of solution were then injected through a Millipore filter sterilizer unit attached to a five-ml syringe into the liquid agar solution and mixed thoroughly before pouring. Exactly 0.1 ml of a late log culture of Crithidia was then inoculated directly onto nine plates and spread with a glass rod. This procedure was repeated for each concentration used. The undefined medium containing 15 µg/ml hemin was used in these experiments. After 9-12 days, colony counts were made.

	Concentration 4-NQO (µg/ml)					
	0	0.1	0.2	0.3	0.4	0.5
Number of colonies/plate	300	200	50-80	10-50	5-10	1-5

Although the ratio of white to red colonies steadily increased after the 5th selection, there were no changes in rate of O_2 uptake of those cells when measured polarographically. By the 9th selection, practically all the colonies were white and appeared smaller than the controls. One colony was serially subcultured over several months. After replating, it retained both identifying characters: small size and an inability to reduce tetrazolium. The progeny of this clone was used in the studies which follow.

At the outset it was important to determine whether the TTC-negative strain was induced or selected. Three techniques are commonly applied to distinquish between these two alternative: (1) exposure on media inhibiting growth of respiratory-deficient clones such as lactate medium; (2) application of the Delbruck-Luria fluctuation analysis; and (3) testing for the acquisition of resistance to the inducing agent. For technical reasons we chose (3) and found that the TTC-negative strain was as sensitive to 4-NQO as the controls, implying but not proving that induction rather than selection had occurred.

b) Growth Rate Differentials

During the selection procedure the white clones reached plateau phase later than controls. Therefore, after the 9th subculture on NQO agar from a white colony the growth rates of the TTC-negative strain and controls were compared by turbidimetry, cell counts, and protein content (Charts 9, 10, 11). For clarity some salient features of the curves are listed in Table 12.

Computation of the growth rate constant K (Packer, 1967), defined by the expression:

$$K = 2.3 \frac{(\text{Log } N - \text{Log } N_o)}{T - T_o}$$

showed that on the basis of protein and turbidity measured between 20-30 hr differences in K of an order of magnitude were found between TTC-negative and control strains. However, if cell number was measured during the same time interval K showed insignificant differences. Possible reasons for this discrepancy will be discussed later. As shown on Charts 9, 10, and 11 and Table 12 the mutant population eventually reached the same levels as controls although these levels were reached approximately 30 hr later. When cells were grown in the low-hemin defined medium it was noted that under these restrictive conditions not only was growth slowed

but the mutant population was unable to reach the end point values attained by the controls. This is shown graphically in Charts 12, 13 and in tabular form on Table 13. Under these conditions comparison of mid-point and K values reveals similar differentials as those in undefined medium (15 µg/ml).

c) Colony Size Differentials

When replated after 6 months of continuous subculture in defined medium, 98% of the colonies appeared small and white under TTC agar overlay. Measurement of colony diameters at this time showed that the mutant colonies were smaller than control colonies (Table 14), in terms of colonial growth (increasing concentric circles) paralleling the differentials in liquid media. The difference between the two means of colony diameters was not statistically significant, mainly because of the variability of both populations. More measurements would be necessary to compensate for this variability.

LEGEND CHARTS 9, 10, 11

Two flasks containing 100 ml undefined medium (15 µg/ml hemin) were inoculated with control and mutant cells respectively; one ml/aliquots were taken for protein, cell count, and turbidity measurements at 8, 26, 30, 50, 58, 74, and 84 hr. Aliquots were in duplicate. Similar results were obtained using defined medium (15 µg/ml hemin).

CHART 9

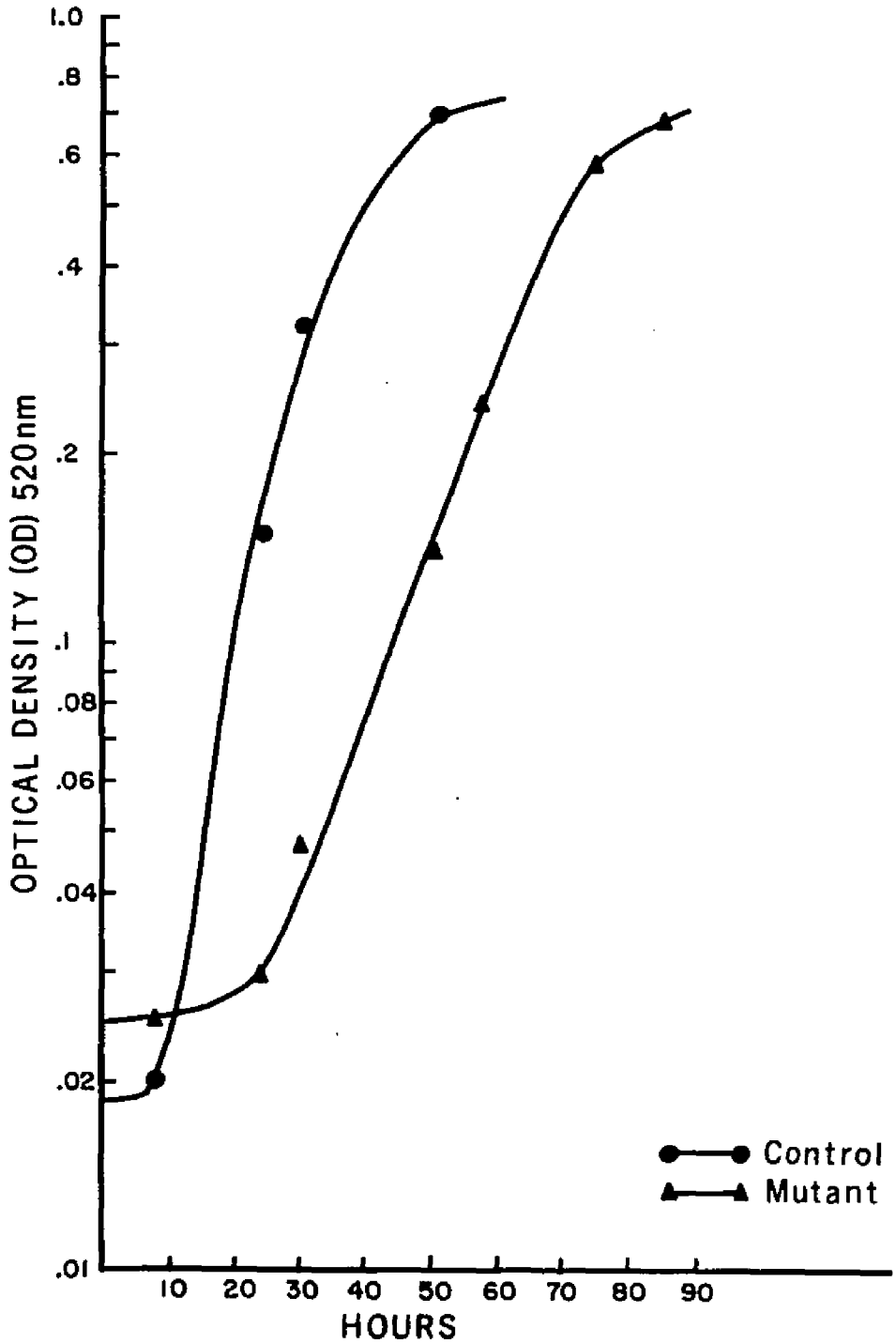

1.0
.8
.6
.4
.2
.1
.08
.06
.04
.02
.01
OPTICAL DENSITY (OD) 520nm
10
20
30
40
50
60
70
80
90
HOURS
Control
Mutant

CHART 10

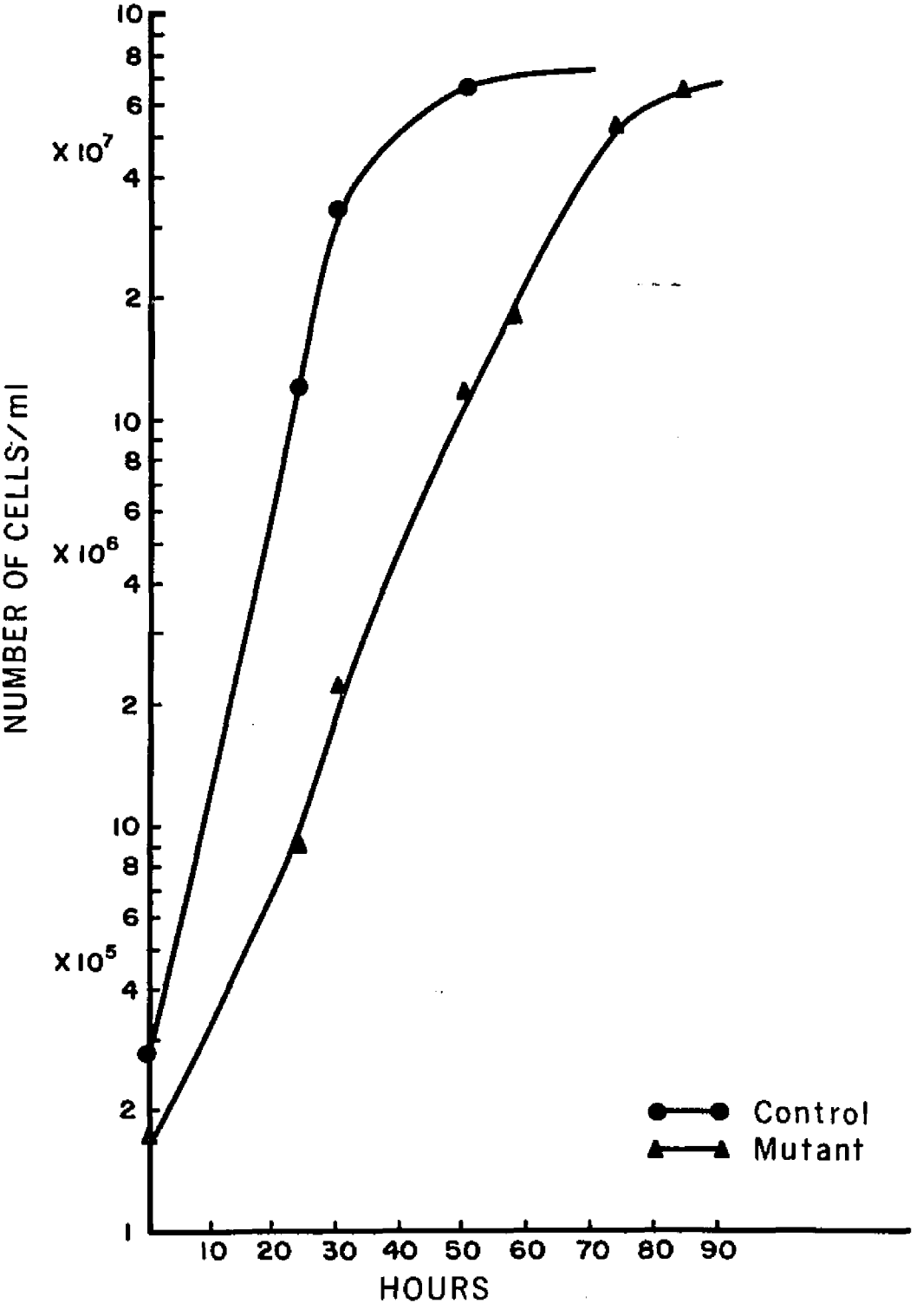

NUMBER OF CELLS/ml
X 10⁷
X 10⁶
X 10⁵
10
8
6
4
2
1
10 20 30 40 50 60 70 80 90
HOURS
Control
Mutant

CHART 11

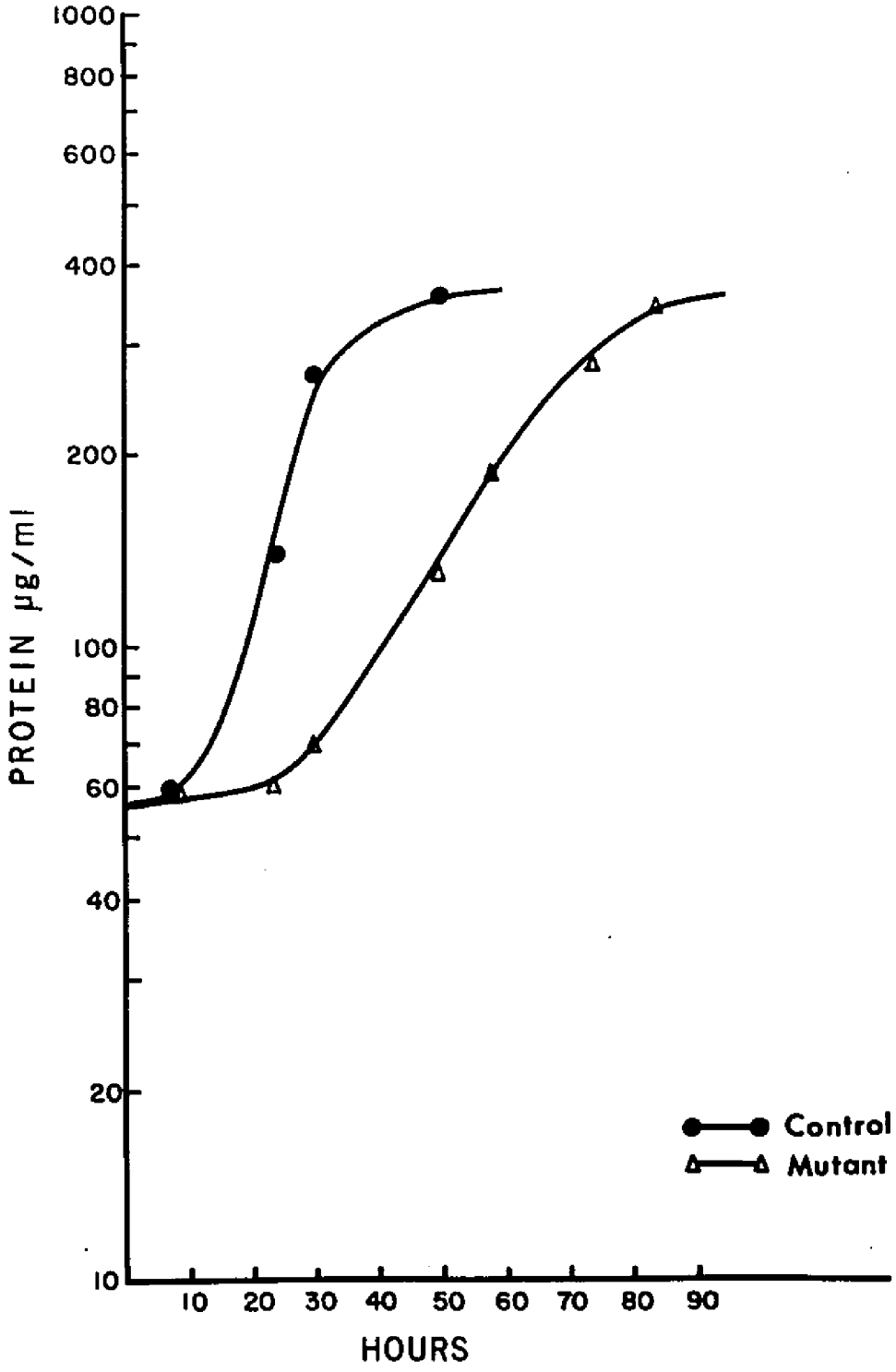

1000
800
600
400
200
100
80
60
40
20
10
PROTEIN µg/ml
10 20 30 40 50 60 70 80 90
HOURS
Control
Mutant

TABLE 12

Growth Characteristics of TTC-Negative Strain in Undefined Medium.

K was calculated between 20-30 hr under each condition; mid-point and end-point of control was selected for comparison with mutant.

	K (20-30 hr)		Mid-Point of Control (20 hr)		End-Point	
	Mutant	Control	Mutant	Control	Mutant	Control
Absorbance	0.0025	0.040	0.028	0.110	0.700	0.700
Cells/ml	0.114	0.155	7×10^5	7×10^6	68×10^6	68×10^6
Protein µg/ml	0.0145	0.740	58	95	350	360

LEGEND CHARTS 12, 13

Mutant and control cultures were grown in duplicate at 27 C, in defined medium containing 2 µg/ml hemin in 100 ml "nephela" culture flasks. Aliquots for cell count were taken and turbidity measured at 0, 16, 25, 39, 48, 66, 90 hr. Each 1 ml aliquot was in duplicate.

CHART 12

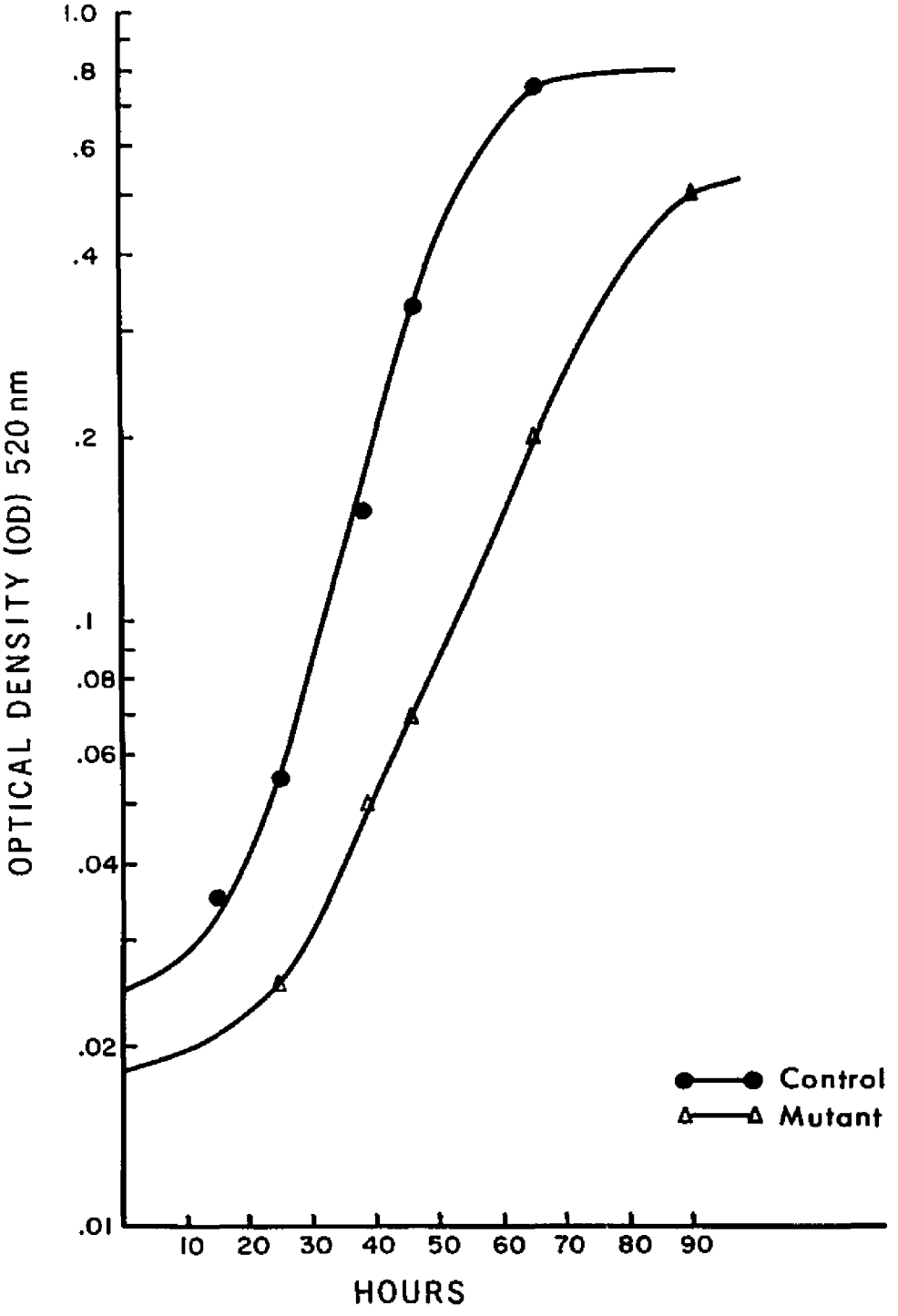

1.0
.8
.6
.4
.2
.1
.08
.06
.04
.02
.01
OPTICAL DENSITY (OD) 520 nm
10
20
30
40
50
60
70
80
90
HOURS
Control
Mutant

CHART 13

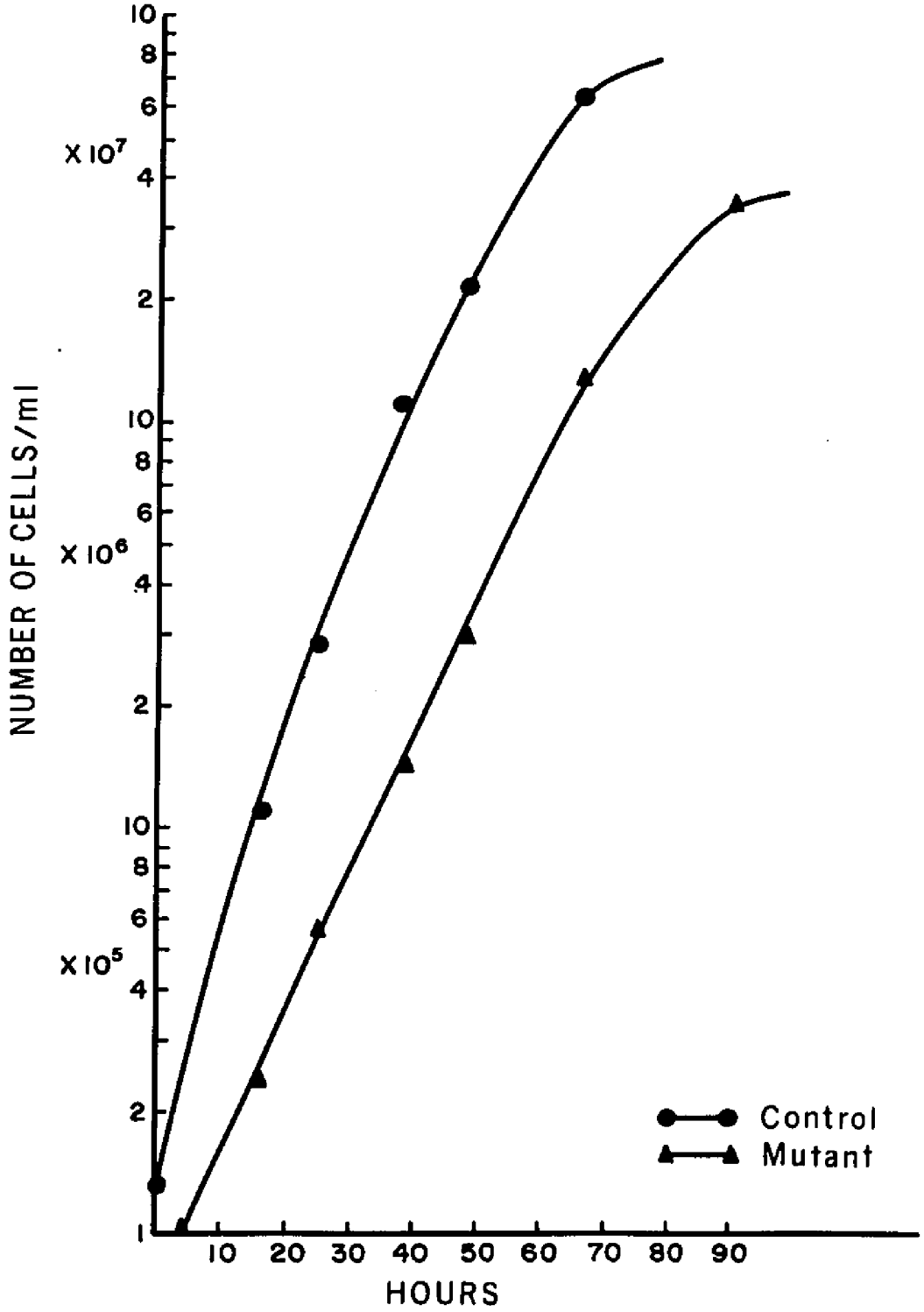

NUMBER OF CELLS/ml
X 10⁷
X 10⁶
X 10⁵
10
8
6
4
2
10
8
6
4
2
10
8
6
4
2
1
10 20 30 40 50 60 70 80 90
HOURS
Control
Mutant

TABLE 13

Growth Characteristics of TTC-Negative Strain in low-hemin defined medium.

Cells were grown in defined medium containing 2 μg/ml hemin and measurements taken as described in Table 12.

	K (30-40 hr)		Mid-Point of Control (40 hr)		End-Point	
	Mutant	Control	Mutant	Control	Mutant	Control
Absorbance	.0046	0.0295	0.052	0.220	0.56	0.75
Cell number/ml	0.0943	0.1150	1.8×10^6	11×10^6	3.5×10^7	6.8×10^7

TABLE 14

Comparison of Colony Diameters: TTC-negative vs control.

Cells were plated on defined media containing 1.25% agar and on day 7 TTC overlay was performed. Colony diameters were measured under a light microscope equipped with a Bausch and Lomb stage micrometer.

	Comparison of Colony Diameters: Mutant vs Control	
	Mutant	Control (in stage micrometer units)
Mean	2.20	3.12
Variance	.599	.592
SD	.77	.77
n	26	26
t	0.64	
p	0.75 (not significant)	

d) Analysis of Respiratory Pigments

Although the ability to reduce TTC was impaired, an associated decrease in respiration was, surprisingly, not demonstrable by O_2 uptake. Uptake rates varied widely from sample to sample and were never lower than 80% of controls; respiration was sensitive to azide and cyanide, indicating that the terminal oxidase was intact, nor did spectrophotometric analysis of respiratory pigments reveal differences.

Difference spectra of mutant and control mitochondrial preparations were used instead of absolute spectra to determine alterations in respiratory pigments for the following reasons:

a) difference spectra are based on the fact that the and β bands of ferro-cytochromes are detectable in the reduced but not in the oxidized state (Chance, 1954). This eliminates interference from other heme-containing proteins such as catalase and cytochrome $\underline{b}_5$ which cannot be readily oxidized or reduced.

b) furthermore, since difference spectra are performed on mitochondrial fractions prepared from whole cells, unwanted cellular contaminants can be washed out during the preparative procedure.

c) difference spectra are highly reproducible and provide sharply defined absorption maxima.

An electron micrograph of the crude mitochondrial fraction prepared as described in "Methods" is shown in Fig. 17. The pellet consist of numerous flagella, membranous components, debris, and swollen, partially broken mitochondrial fragments. Intact kinetoplasts were sometimes seen. At no time were intact microbodies seen.

As shown in Chart 14, in agreement with Hill & Anderson (1970), the following cytochromes were present in Crithidia: cytochrome b (maximum at 560 nm) and β and Soret bands respectively at 527 and 430 nm. Cytochromes a + a_3 with a characteristic band at 605 nm; a Soret band seen as a hump, at 444 nm is also present. Cytochrome c with an a maximum at 553 nm is not apparent in our spectra. This, however, does not imply that cytochrome c is absent. Hill & Anderson (1970) showed that cytochrome c is demonstrable at -190 C; we did not carry out low-temperature observations, hence we assume from Hill & Anderson's results that cytochrome c is present but undetectable by our methods. Incubation of the mitochondrial preparation with dithionite for more than 30 min did not reduce cytochromes any further.

LEGEND FIG. 17

Electron Micrograph of Crude Mitochondrial Preparation. The crude mitochondrial pellet was sedimented at 16,318 g and fixed overnight in 3% glutaraldehyde. It was then washed twice in cacodylate buffer, stained in 2% uranyl acetate, dehydrated in an acetone series, infiltrated with 1:1 Epon: acetone for 5 min, then pure Epon (twice). The pellet was then embedded in fresh Epon for 24 hr at 60 C. The remaining steps are described in "Methods". Note flagella (fl) in transverse and longitudinal section and swollen mitochondrial fragments (Mf) inside of which cristae (cr) can be seen. X 28,200


17
M1
fl
Mf
Tu

LEGEND CHART 14

Difference Spectra: TTC-Negative Strain vs Controls. The lines represent the difference in absorption between a crude mitochondrial fraction in which respiratory pigments were reduced by addition of sodium dithionite to a portion of the preparation in which the respiratory pigments were oxidized by aeration for 15-30 min. The mitochondria were suspended in sucrose buffer at concentrations of 1.66 and 3.0 mg protein/ml respectively for mutant and controls; temperature 25 C; light path of the cuvettes 10 mm.

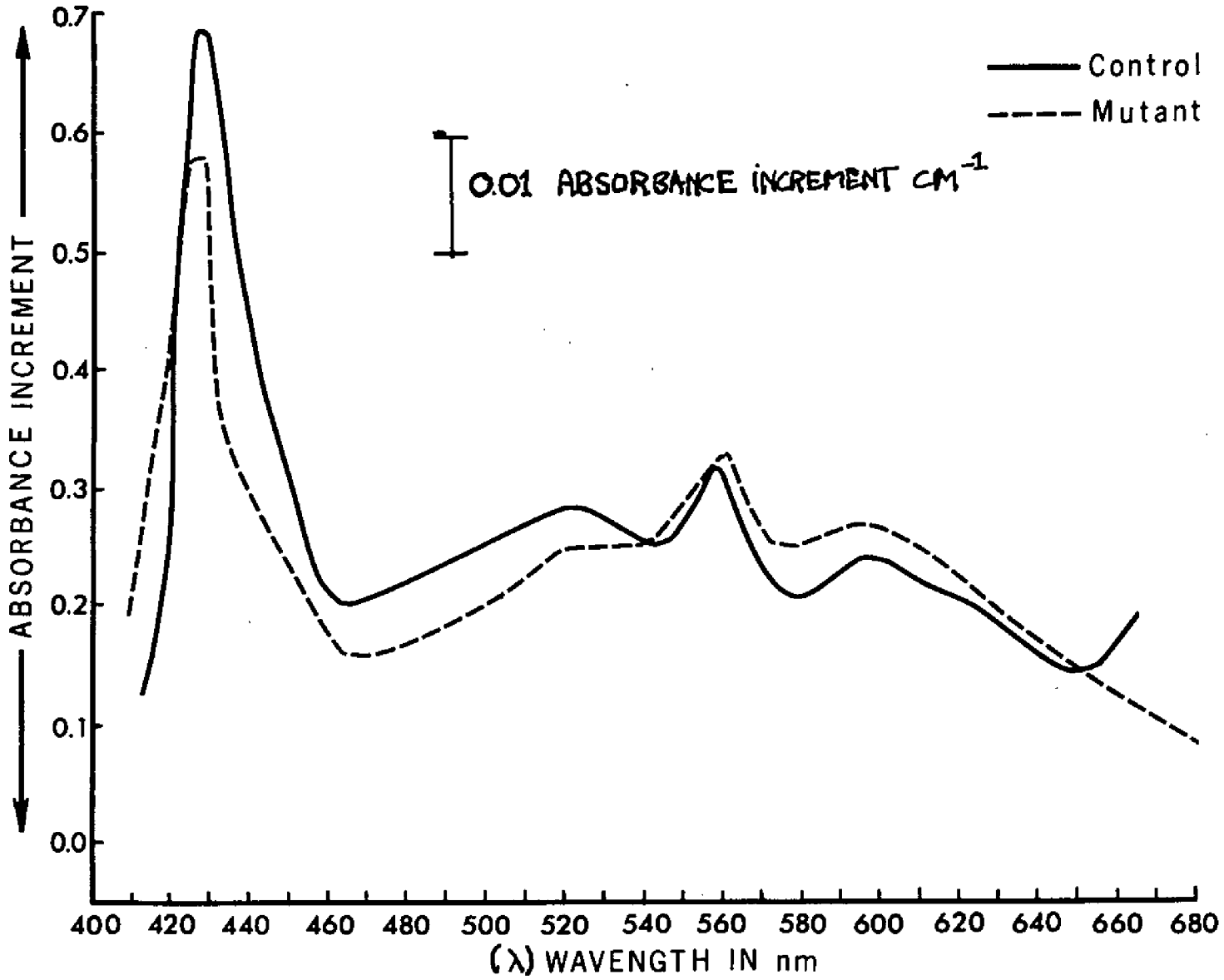

Control
Mutant
0.01 ABSORBANCE INCREMENT CM-1
ABSORBANCE INCREMENT
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
400 420 440 460 480 500 520 540 560 580 600 620 640 660 680
(λ) WAVENGTH IN nm

CHART 14

The difference spectra (Chart 14) indicated no significant differences in respiratory pigments between mutant and controls; the bands which are diagnostic for cytochromes showed close similarities. We conclude therefore that the mutant strain is not grossly deficient in cytochromes and that O_2 consumption is not significantly impaired despite the strain being TTC-negative.

e) Ultrastructural Changes

Inspection of electron micrographs of mutant cells revealed no alterations in the nucleus or nucleolus; however the cytoplasmic microbodies appeared to have increased in size and number. To determine in a quantitative fashion whether the relative volume ratio of the microbodies to the whole cell had indeed increased in the mutant, the technique of morphometric analysis was used. The principles on which the application of this technique to biological materials is based are discussed in detail by Baudhuin (1968). Briefly, this technique is based on the assumption that quantitative information can be obtained about cell components in three dimensional terms from two dimensional representations such as an electron micrograph. Accordingly to the

principle of Weibel (1969), which is expressed symbolically as:

$$V_{Vi} = A_{Ai} = L_{Li}$$

the fractional volume occupied by a structure is equal to its fractional area in a random cross-section or the fractional length of a transecting random line. Thus the volume of an organelle which appears as an area in cross section can be measured by lines traversing the cross-sectional surface. The areas are measured in practice by superimposing an array of sampling lines over the micrograph and measuring the lengths of the lines transecting the organelle (Fig. 16). A sample data sheet is shown in Table 15. Note that some lines traverse neither of the cytoplasmic organelles some both and some only one organelle. This is visualized in Fig. 16. The results of quantitative morphometry are shown in Table 16. Clearly, an increase in the average volume of the microbodies was evident in the mutant population in comparison to controls, whereas no substantial change is found in mitochondrial volumes or in whole cell volumes. The latter has been corroborated by Coulter counter measurements (Chart 15).

LEGEND FIG. 16

Electron Micrograph of Crithidia as it Appears in the Measuring Frame For Morphometry. A grid of 17 vertical lines is superimposed over the micrograph. Reproduced at 1/2.5 actual size. Plate * Z 1495.

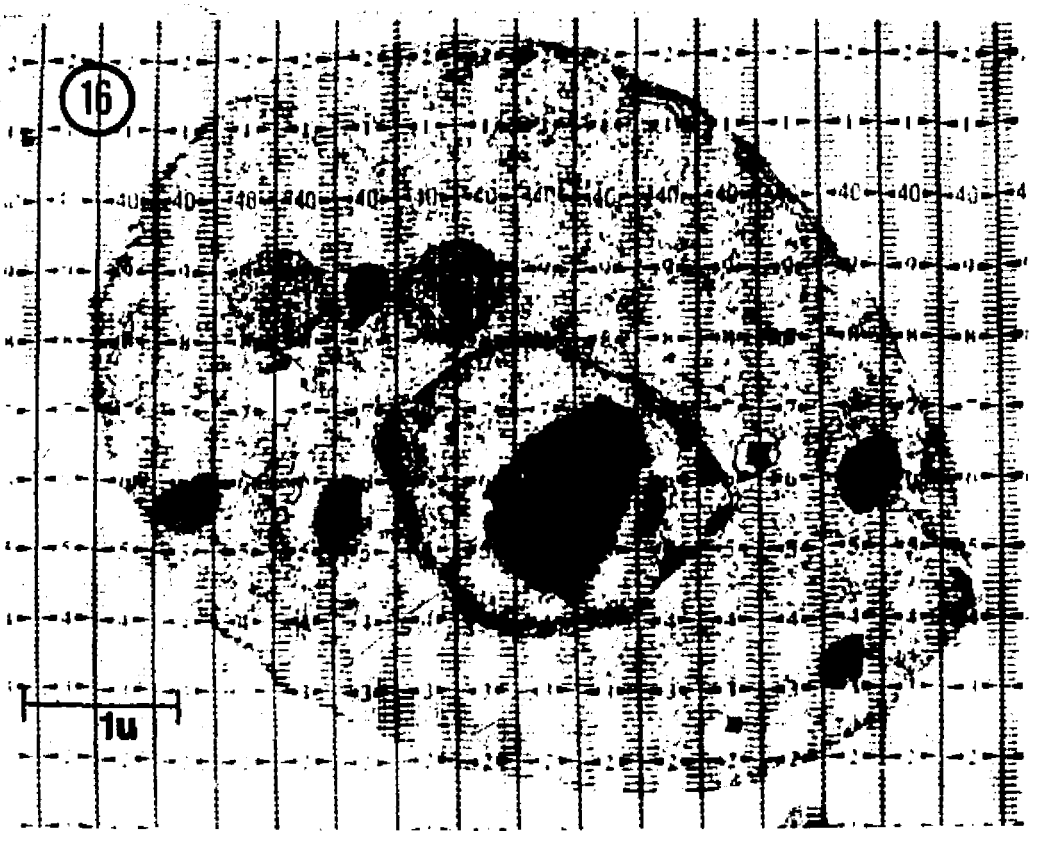

16
1u

TABLE 15

Sample Data Sheet for Quantitative Measurement of Cell Structures. Sample data sheet of an individual cell subjected to morphometric analysis. Values along horizontal rows represent measurements of organelles transected by vertical sampling lines in mm.

Subject : Crithidia fasciculata

Plate No. : Z 1519

Print Magnification: 19,500X

Column	Whole Cell (mm)	Mitochondria (mm)	Dense Bodies (mm)
1	22	13	
2	36	10	
3	47	7	
4	57		10
5	65		12
6	73		
7	80		
8	82	8	12
9	90	3	
10	70		
11	90		
12	90		20
13	85	8	
14	78	10	
15	70		4
16	58		9
17	40		
	1,223	59	67

TABLE 16

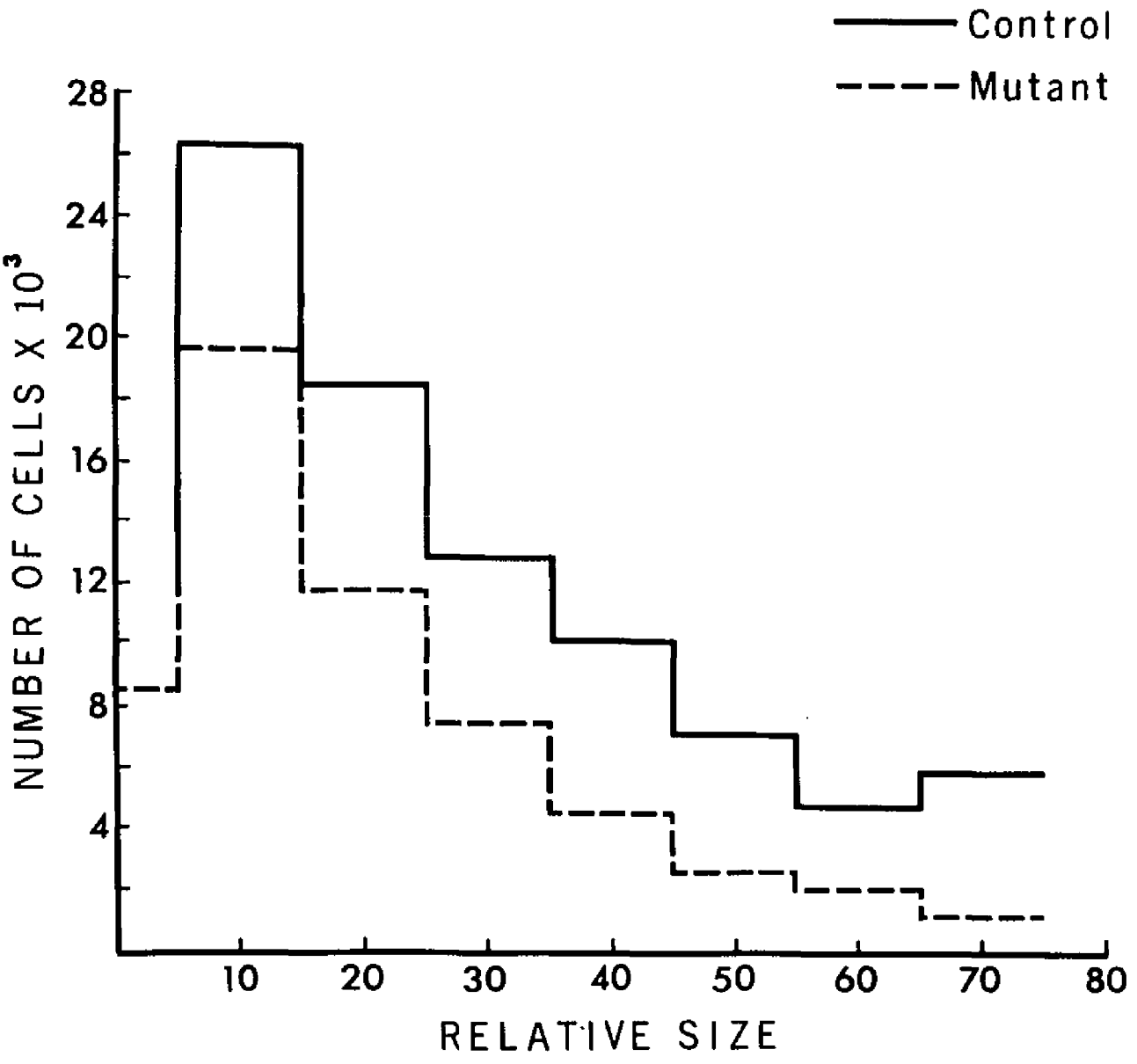
Statistical Analysis of Morphometric Data. Mean ($\bar{x}$), standard deviation (SD) and standard error (SE) computed by averaging morphometric data obtained from N cells.

	$\bar{x}$	SD	SE	N
		Control		
Whole Cell	839.8	±317	46	45
Mitochondrion	50.6	±30.2	4.5	45
Microbody number/cell	1.6	±1.3	0.184	45
Microbody volume	20	±16.5	2.47	45
		Mutant		
Whole Cell	955	±312	61	27
Mitochondrion	48.8	±25.9	5.2	27
Microbody number/cell	2.6	±1.9	0.375	27
Microbody volume	22	±22	4.3	27

LEGEND CHART 15

Frequency Histogram: Cell Number/Volume Ratios of TTC-Negative and Control Strains. Coulter-counter measurements were performed on late log cultures of both populations grown in undefined medium containing 2 µg/ml hemin.

CHART 15


Control
Mutant
NUMBER OF CELLS X 10^3
28
24
20
16
12
8
4
10
20
30
40
50
60
70
80
RELATIVE SIZE

In control cells the per cent of total cell volume occupied by the mictochondrion was calculated to be 6% while that occupied by the microbodies was 2.35%. In contrast, mutant cells showed a microbody/whole cell ratio of 4.83%, more than twice that of controls. The relative mitochondrial volume was 5.05% of the whole cell volume.

On the basis of the student t test the difference between the two means was insignificant meaning that such variation could have occurred by chance alone (Table 17). However, when the data was treated according to the method of least squares, regression lines with significantly different slopes were computed (Chart 16). By comparing the values for the means in Chart 16 with those in Table 16 the correctness of the regression lines was confirmed. It can be seen that $\bar{X}$, $\bar{Y}$ calculated by the least squares method agrees with $\bar{X}$, $\bar{Y}$ computed from pooled data.

f) Catalase Assay and Localization

Assay of catalase by the method of Baudhuin (1968) revealed that Crithidia possesses large amounts of this enzyme; moreover, in comparison to other tissues and organisms Crithidia has the highest specific activity of any cell type yet studied. The catalase activity of various protozoa can be seen in Table 18.

TABLE 17

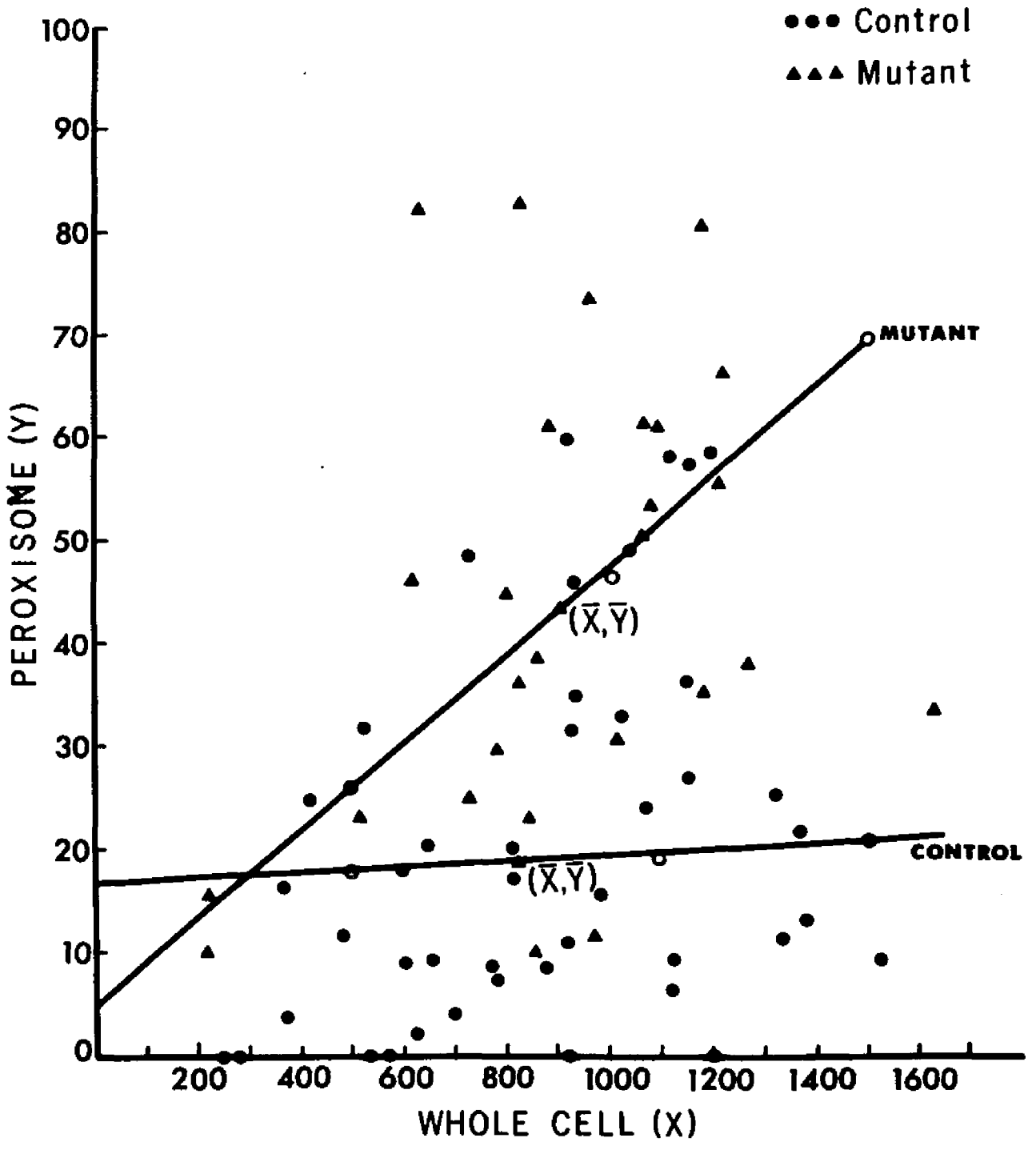
t Test for Significance of Means. t and p values computed from data tabulated in Table 16.

	t	p
Whole Cell	0.448	0.9
Mitochondrion	0.51	1.0
Microbody Number	0.435	0.75
Microbody Volume	.882	0.10

LEGEND CHART 16

Scatter Diagram with Regression Lines Superimposed Illustrating Least Squares Fit. Scatter diagram relating whole cell (X) to peroxisome content (Y) computed from samples of mutant and control populations. Regression lines of Y (peroxisome) on X (whole cell) fitted by the least squares method are superimposed on the scatter diagram.

CHART 16


●●● Control
▲▲▲ Mutant
MUTANT
CONTROL
(X̄,Ȳ)
(X̄,Ȳ)
PEROXISOME (Y)
WHOLE CELL (X)
0
10
20
30
40
50
60
70
80
90
100
200
400
600
800
1000
1200
1400
1600

Since catalase is the single constant component of the class of microbodies termed peroxisomes (Muller, 1969) we hypothesized that Crithidia catalase was confined within the boundaries of the microbody. Localization of catalase, or any enzyme, in a specific organelle can be demonstrated in two complementary ways (a) by cell fractionation techniques combined with enzyme assays for particle-bound catalase or, (b) by ultrastructural cytochemistry, a technique in which a chemical reacts with hydrogen peroxide in the presence of catalase producing an electron opaque product localized within the microbody (Seligman et al., 1968).

We were unable to demonstrate particle-bound catalase by cell fractionation techniques. Methods based on shear forces, nitrogen cavitation (Hunter & Commerford, 1961) enzymatic digestion or swelling in hypotonic solution (Simpson, 1968b) were equally unsuccessful. In each case catalase activity was found in the supernatant indicating either (a) that catalase was soluble in the cytoplasm or, (b) that the microbody wall was broken during the fractionation procedure and catalase leaked out. Other techniques such as sonic oscillations and

the French pressure cell were not attempted. Since we were unable to demonstrate sedimentable catalase in Crithidia, we turned to ultrastructural cytochemistry ("Methods").

A photomicrograph of Crithidia after reaction was DAB oxidation medium taken under phase contrast is shown in Fig. 10. Included for comparison is a Giemsa-stained preparation of Crithidia taken at the same magnification (Fig. 11). The Giemsa stain, which is strongly acidophilic, is taken up primarily in the kinetoplast region (K) and the nucleus (N). In contrast, the DAB oxidation product is localized in discrete cytoplasmic regions in a random fashion very reminiscent of microbody distribution (mi).

The results of light microscopy were substantiated by electron microscopy (Figs. 12, 13, 14, 15). The dense aggregations of DAB oxidation product are unmistakably localized in the microbodies (mi). DAB is also taken up by the mitochondrial cristae (cr), though to a lesser extent, due to interaction with the heme-containing cytochromes.

The evidence presented strongly suggests that the cytoplasmic organelles previously known as "dense bodies" (Hill et al., 1968) actually belong to a biochemical sub-class of microbodies known as peroxisomes.

TABLE 18

Comparison of Catalase Activity in a Variety of Protozoa.

Organism	Specific Activity (mUnits)
Rhizopoda	
Amoeba proteus	1.4
Chaos chaos	0.2
Acanthamoeba	79
Flagellates	
Ochromonas malhamensis	1.7
Crithidia fasciculata	516
Ciliata	
Tetrahymena pyriformis	33

[Adapted from M. Muller (1969.]

Fig. 10. Photomicrograph of Crithidia fixed in calcium formol solution and stained with DAB oxidation medium pH 9 at 37 C for 1 hr, taken with a phase-contrast microscope. Note the darkly stained areas located in the cytoplasm showing characteristic form and distribution of microbodies (mi). X 4,550

Fig. 11. Photomicrograph of Crithidia fixed as in Fig. 10 and stained with Giemsa, taken with a phase-contrast microscope. Note the unstained flagellar pocket (fp) adjacent to which can be seen the darkly staining kinetoplast (K). The nucleus is seen as a darkly stained somewhat irregular body several times larger than the kinetoplast (N). X 4,550

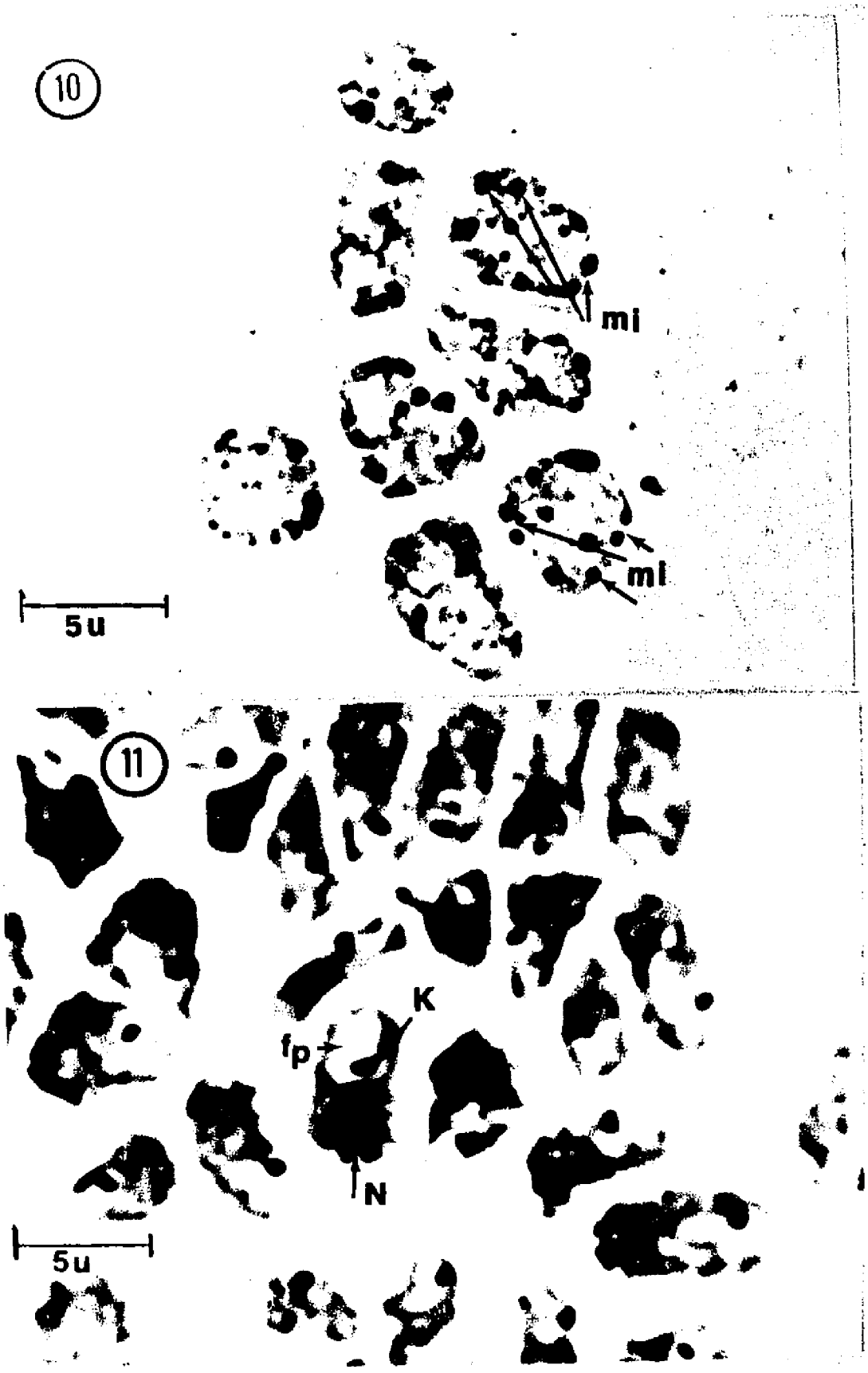

10
mi
mi
5u
11
K
fp
N
5u

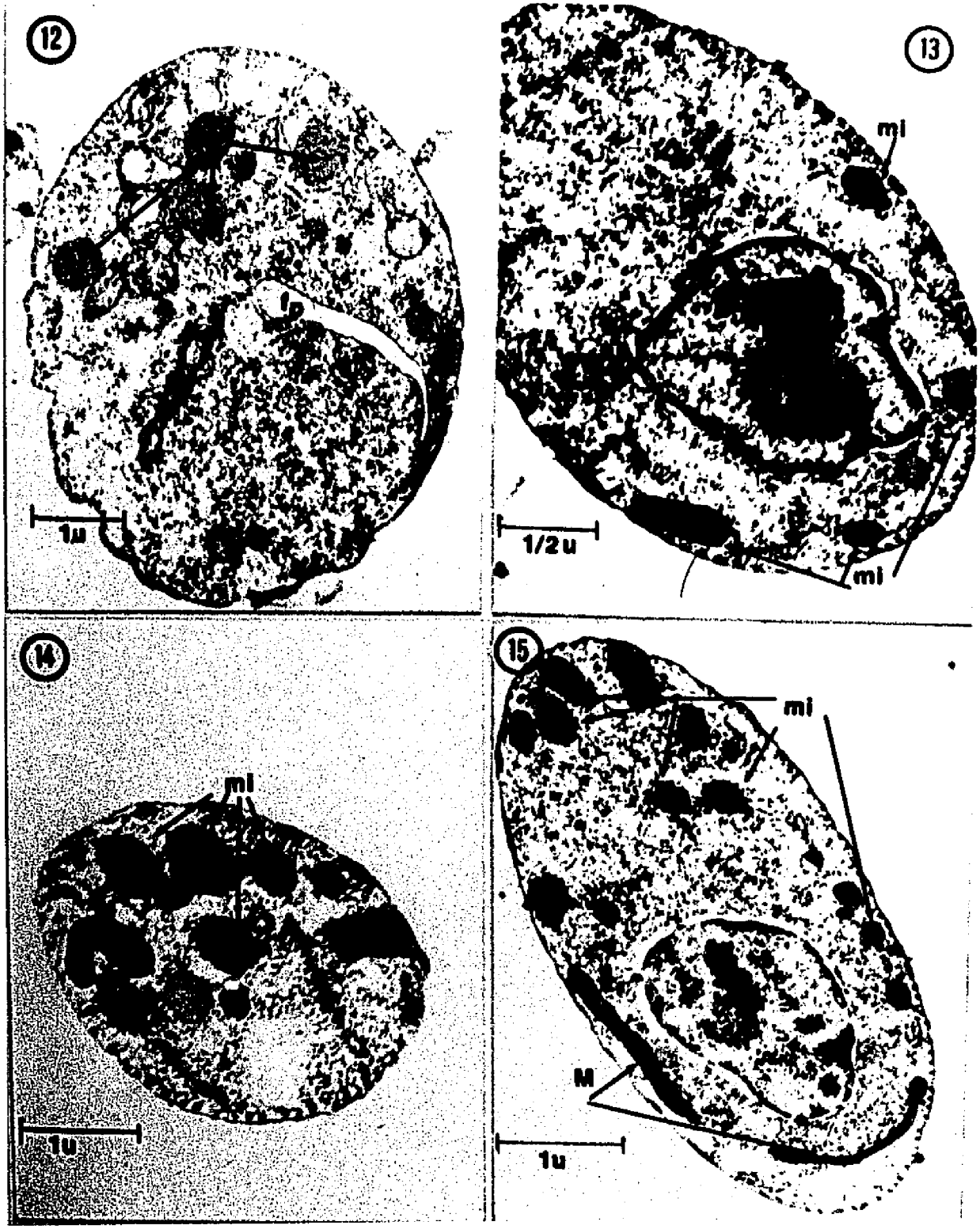
Fig. 12. Transverse section anterior to nucleus of Crithidia fixed with glutaraldehyde and incubated in DAB oxidation medium minus DAB for 1 hr at 37 C. Note the unstained microbodies (mi) with diffuse matrix in the cytoplasm. Sub-pellicular microtubule layer can be seen along left margin of cell. Flagellar pocket (fp) and mitochondrial sections (M) can also be seen. X 16,250

Figs. 13, 14, 15. Electron micrographs of DAB-stained Crithidia.

Fig. 13. Semi-oblique section through Crithidia fixed with glutaraldehyde and stained with DAB medium pH 9, for 1 hr at 37 C. Note densely stained microbodies (mi) along cell periphery. Not all microbodies are stained. X 36,000

Fig. 14. Transverse section through posterior end of Crithidia. Note the numerous microbodies (mi) with characteristic oval contour. Crescent-shaped structure is a mitochondrial section. X 22,000

Fig. 15. Longitudinal section through Crithidia showing numerous stained microbodies (mi); uptake of stain is also seen in the mitochondria (M). X 22,600


12
1µ
13
mi
1/2 u
mi
14
mi
1u
15
mi
M
1u

Since the average volume of the peroxisomes had apparently increased in the TTC-negative strain, catalase assays were carried out to determine whether a parallel increase in enzyme activity had also occurred. The results, recorded in Table 19, show that catalase was substantially higher in the TTC-negative strain (1.29X). Moreover, it can be seen that the absolute amount of catalase is strongly influenced by the concentration of hemin in the medium. Growth in 2 μg/ml hemin yielded distinctly lower catalase activities than growth in 15 μg/ml hemin (Table 18, Table 19).

g) Characterization of Peroxisomes

We further characterized Crithidia peroxisomes by studying the effect of 2,4-aminotriazole (AT) a potent inhibitor or renal and hepatic catalase, on the Crithidia enzyme. As seen in Table 20, catalase isolated from AT-treated cells showed lower activities than untreated controls. However, if AT was added to mid-log phase cultures no inhibition of growth occurred.

Since catalase is usually found associated with one or more oxidases in the peroxisome (de Duve, 1969) we applied the ^{14}C-formate

method described by de Duve (1966) to determine which oxidases were present in Crithidia peroxisomes. The following substrates were tested in cell-free suspensions and intact cells: D-alanine, urate, glycolate, L-alanine, lactate, hypoxanthine, and -glycerophosphate. Under the conditions used, we were unable to demonstrate any of the usual oxidases found associated with catalase in other organisms. Hence, at present, only a single enzyme, catalase, has been demonstrated in Crithidia peroxisomes and this by cytochemical methods only.

TABLE 19

Catalase Activity of TTC-Negative Strain. Assay carried out on the same population used for morphometric analysis. Cells were grown in defined medium containing 2 µg/ml hemin; at 48 and 92 hr respectively control and mutant cells were prepared as described in "Methods" for catalase assay. Each assay was done in duplicate.

	Mutant	Control
Protein µg/ml	420	495
Catalase activity (mUnits)	420	326

TABLE 20

The in vivo Effect of Amino Triazole on Catalase Activity in Crithidia.

After 28-hr growth three cultures of Crithidia were exposed to 0, 1×10^{-3} and 5×10^{-3} M AT incubated at 27 C and harvested at 47 hr. The fractionation procedure and catalase assay were carried out as described in "Methods".

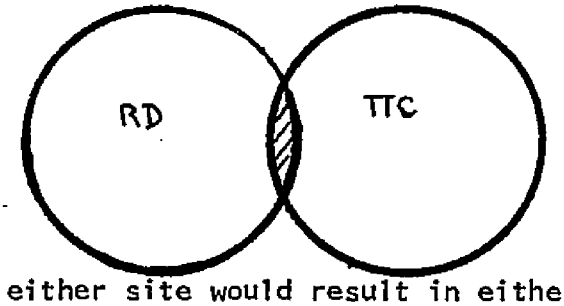
Condition	Catalase Activity (mUnits)
Control	550
AT 5×10^{-3} M	184
AT 1×10^{-3} M	560

DISCUSSION

Several properties exhibited by the TTC-negative strain are shared with conventional RD mutants, i.e., inability to reduce tetrazolium and slow growth; however, the presence of the usual cytochrome spectra and rates of O_2 consumption suggest strongly that this is not a conventional RD mutant (Ngai et al., 1961). Therefore we have named it TTC-negative to set it off from yeast petites, and protozoan RD mutants (Gause, 1966). The TTC-negative trait apparently has a genetic origin; the term "genetic" being used loosely here because conventional Mendelian crosses cannot be carried out with Crithidia; it is genetic only in the sense that information for "slow growth" is passed from parent to daughter cell at each mitosis.

It appears paradoxical at first that a TTC-negative strain should consume O_2 at the same rate as the parent strain especially since tetrazolium reduction is the prime selective criterion for RD mutants in yeast. However, this paradox resolves on realization that the correlation between tetrazolium reduction and impaired respiration is based on empirical observations in only a few species of yeast (Ogur

et al., 1957). Hence one should not assume that a TTC-negative colony is of necessity respiratory deficient. Our results become understandable if we assume that the TTC reaction is spatially separate from the site responsible for respiratory deficiency. In the diagram below each circle denotes an enzyme or multienzyme complex.



A lesion at either site would result in either a TTC-negative clone or a respiratory-deficient one. A lesion in the hatched area, on the other hand, would result in a single clone exhibiting both properties. Yeast petites represent the latter, while the Crithidia TTC-negative strain exemplify the former. Localization of the TTC reaction at lipoyl dehydrogenase rather than at the cytochrome chain supports this interpretation of our results (Hill & Anderson, 1970).

The TTC-negative strain exhibits several properties similar to mammalian cells transformed by 4-NQO, e.g., Sato et al. (1970) reported that respiration in 4-NQO transformed cells was similar to controls with glucose as the sole carbon source and higher than controls when pyruvate

served as sole energy source. They also showed that transformed cells had a consistently higher rate of aerobic glycolysis; and the CO_2 ratio of C_6/C_1 -- a useful marker for evaluating the relative participation of the TCA cycle and pentose shunt -- showed no changes; in many tumors this ratio decreases. These findings suggest that the TTC-negative strain may serve as a counterpart of cancer in Crithidia. Further biochemical analysis along the lines of Sato's work might provide better evidence for this statement.

Another parallel between the TTC-negative strain and transformed mammalian cells is the high sensitivity shown by the cells toward the inducing agent (Kakunaga & Kamahora, 1968; Kuroki et al., 1967; Kuroki et al., 1968; Namba & Sato, 1970). Cells transformed by carcinogenic hydrocarbons, in contrast, are highly resistant to the inducing agent (Haddow, 1938b). One must therefore entertain the idea that important differences underlie the action of these two classes of carcinogen.

In Table 12, 13 the growth rate constant K, differs depending on method of measurement, reflecting the technical limitations inherent in each method. For example, if a cell is extremely massive at a given

stage, i.e., a high mass:volume ratio, it will tend to show a high protein:cell number ratio. Should the m:v ratio fluctuate during the growth cycle, correlation between cell number and protein values will be poor. Furthermore, turbidimetric measurements are flawed by the fact that they are based primarily on light scattering effects which in turn are based on the assumption that solute particles are in a dilute, ideal solution and behave as independent scatterers as do particles of an ideal gas -- an assumption which can hardly apply to suspension of Crithidia (Born, 1970). Interference between scattering centers in an individual cell and the effect of high densities are among the many unknown factors influencing turbidity measurements. Nonetheless, we can draw two general conclusions from our data: (1) that entry into log phase from lag phase is slowed and, (2) that the log phase increment per unit time is diminished in the mutant. The discrepancy between K calculated for cell number, protein and turbidity could be explained if one assumes that during lag phase mitosis continues while the total mass of the cell population remains unchanges. This would require that turbidity measurement was dependent on total cell mass and was independent of cell number -- an assumption that remains to be proven.

The slowed growth observed contradicts the well-worn idea that cancer involves "unrestrained" growth (if one accepts the preceding argument that the Crithidia mutant corresponds to a cancer cell). How legitimate, therefore, is it to regard the TTC-negative strain as representing a protozoan counterpart of a cancer cell? A sidelight was provided by Baserga & Keisieleski (1962) who showed that tumor cells do not necessarily proliferate faster than normal cells. Cancer cells are now believed to have their controls for cell division set at different levels than normal cells, rather than being "out of control" (Baserga, 1971). Hence the TTC-negative strain may be regarded as a strain whose control system is set for a lower rate of cell division that its parent strain.

A damping effect, defined here as reduction in final cell number, is shown by the TTC-negative strain grown in low-hemin but not high-hemin defined medium (Table 13). Such a response suggests that the new metabolic system of TTC-negative strain has somehow acquired a higher sensitivity to the concentration of exogenous hemin than the parent population. Precisely how regulation of heme metabolism is involved

in the mutant phenotype is unknown at present.

We were unable to fractionate Crithidia for microbodies without breaking the microbody membrane, no doubt owing to the unusually tough outer cell wall which is reinforced by a sub-pellicular layer of microtubules (Angelopoulas, 1970). With T. equiperdum however, Bayne et al. (1969) did make a crude preparation of microbodies; these bodies, presumably containing the cyanide-insensitive GPO, were obtained after breakage in the French pressure cell. Microbodies were not demonstrated cytochemically nor have they been characterized with respect to other oxidative enzymes, e.g., catalase, D-amino acid oxidase, etc.

The statistical problems inherent in morphometric studies of cell structures are detailed by Weibel (1969). Foremost among these problems is sampling and variability. High variability results from the fact that one measures a sub-sample of a sample: (a) each section is a random slice through the cell and, (b) each section is selected at random on the EM grid. Hence unless large populations are sampled large standard deviations and standard errors commonly result (Table 16). The t test was unsuitable for such an analysis. As an alternative the

method of least squares -- a device to clarifying variability -- was applied. After computing the equations, the slope of the regression line of X and Y of the TTC-negative strain proved to be statistically significant based on a null hypothesis. Chart 16 reveals, however, that the results are open to two possible interpretations: (1) large numbers of peroxisomes may have shifted to the center of the cell and away from the peripheries or, (2) the relative volume of the peroxisomes has increased. Our analysis rules out neither possibility, though the possibility of (1) seems remote.

Little information is available concerning alterations in microbodies under the influence of chemicals or in cells transformed by chemical carcinogens. The hypolipidemic agent, ethyl chlorophenoxy isobutyrate, induces proliferation of hepatic microbodies but little is known about the mechanism (Svoboda & Azarnoff, 1966). Furthermore, in contrast to our findings, the effect disappears upon withdrawal of the agent. Our results with Crithidia show a striking parallel to those of Dalton (1964) and more recently Mochizuki et al. (1971) on peroxisomal alterations in hepatomas; all reported that the size and complexity of peroxisomes

in a series of chemically induced hepatomas was inversely proportional to growth rate. In slow-growing hepatomas, peroxisomes were larger and contained complex crystalloids in the interior in comparison to fast growing strains. Moreover, Mochizuki et al. (1971) observed that several peroxisomal enzymes such as catalase, urate oxidase and D-amino acid oxidases showed higher activities in slower growing strains than in the parent strain.

Since the increase in peroxisome volume reported here correlated with elevated catalase levels, presumably the cell synthesized extra catalase in response to some intracellular demand. Such an increase might be a reaction, expressed as a subcellular compensatory hypertrophy, to an environmental insult. If increased catalase is a protective reaction to 4-NQO, exactly how is it protective?

Interaction between catalase and 4-NQO in vivo may serve to explain the increase in peroxisome volume which we have reported. 4-NQO has been reported to depress cellular catalase presumably by binding to SH groups in the enzyme (Endo et al., 1970). Moreover, we have found that cells grown on 4-NQO-containing agar were more sensitive to 4-NQO in low-hemin media than high-hemin suggesting that some molecules are 4-NQO were

sequestered, inactivated, or modified in such a manner that fewer molecules of 4-NQO would be available to interact with cellular DNA in the ~~low~~ high-hemin medium. If we assume, therefore, that such an interaction occurs and results in the mutual inactivation of catalase and 4-NQO, then, by a process of positive feedback, stimulation of catalase synthesis would result to restore normal catalase levels. After persistant exposure to 4-NQO an over-response to the stimulus might occur characterized by a permanent re-setting of the mechanism which regulates peroxisome volume to a higher level. It can be seen that this hypothetical series of events is open to experimental tests.

The chief gap in our understanding of the increase in peroxisome volume and catalase lies in the current complete ignorance of the role of catalase in the economy of Crithidia. Dr. L. Margulies (pers. commun.) has suggested that catalase may serve to produce O_2 for consumption by the mitochondrion while Crithidia resides in the relatively anaerobic insect gut. Moreover, since kDNA is highly redundant (Simpson, 1972) and may consist of only one or two genes, the possibility has been raised that the large quantities of catalase

found in Crithidia may be coded for by a redundant kDNA gene. If this hypothesis proves true then, in cultures of Crithidia grown in synthetic medium, catalase would serve no function whatsoever. At present there are many loopholes in this hypothesis. For one, although it is true that Crithidia behaves like an obligate aerobe in culture, there has been no convincing evidence that the partial pressure of O_2 is so low in the gut of insects that Crithidia would require a supplemental source of oxygen. Secondly, there is no evidence yet that kDNA codes for catalase protein in Crithidia; and lastly, the source of hydrogen peroxide, the substrate for catalase, must be accounted for as well as the point of oxygen input required for the production of hydrogen peroxide.

A second gap is that only one point in the growth cycle (late log) was chosen in measuring changes in catalase. White (1962) showed in the heme-requiring bacterium Hemophilus and Eeckout (1972) in Crithidia luciliae that catalase levels may fluctuate markedly during the growth cycle. This may hold for Crithidia fasciculata as well suggesting that measurement at two other points in the growth cycle might have been illuminating.

The newly discovered peroxisomes in Crithidia could provide clues to the evolutionary development of the Trypanosomatidae -- one of the most extensive apomictic (asexual) evolutionary developments in the animal kingdom (Ross, 1962). Crithidia is considered a rather primitive member of this family on the basis of (a) simplicity of life cycle and structure (Baker, 1955; Hoare, 1967; Woo, 1970), (b) analysis of fermentation products (Schwartz, 1961) and, (c) high catalase content (Wertlieb & Guttman, 1963). Moreover, Wertlieb & Guttman have proposed that because monogenetic trypanosomes such as Crithidia possess catalase while the more advanced digenetic forms such as T. brucei do not, catalase may serve as a useful phylogenetic marker to trace intermediate stages in the extensive evolutionary radiation of the family Trypanosomatidae.

In higher digenetic trypanosomes organelles closely resembling Crithidia microbodies have been observed (Vickerman, 1969a, b). These structures presumably contain the cyanide-insensitive K-glycerophosphate oxidase (GPO) system which has been characterized biochemically by Grant & Sargent (1960, 1961), Grant et al. (1961) and cytochemically by

Ryley (1966). Though some authors have confused GPO bodies with lysosomes (Malloy & Ormerod, 1971), on the basis of evolutionary conservatism, we consider Crithidia microbodies and GPO bodies to be homologous organelles; see de Duve (1969) and Tolbert (1971) for discussion of the evolutionary relationships of microbodies in plants, multicellular animals and protozoa. Bacchi & Hill (1972) reported substantial amounts of soluble NAD-linked glycerophosphate dehydrogenase activity in Crithidia but did not report any NAD-independent activity which, according to Grant & Sargent (1961), plays a key role in the GPO system. When homogenates of Crithidia were assayed for NAD-independent α-glycerophosphate dehydrogenase, using T. brucei homogenates as positive controls, no activity was found. In sharp contrast, T. brucei homogenates, from either culture or bloodstream forms showed no catalase activity (pers. commun. Dr. A. Balber). Apparently then, the enzymatic complement of the trypanosome peroxisome has changed during the course of evolution from one dominated by catalase to one dominated by GPO.

The loss of catalase in the digenetic hemoflagellates exemplifies Lwoff's (1951) precept that loss of enzymes inevitable accompanies

adaptation to a parasitic life style and may be analogous to the loss of folate -- and hemin -- synthesizing ability (Newton, 1968) which presumably occurred during the earlier transition from a free-living pre-Crithidian to a parasitic life in the insect gut.

Elaboration of a GPO system may reflect a biochemical adaptation to life in a warmblooded host and helps explain the trend towards dedifferention of the mitochondrion in trypanosome evolution. During this transition the GPO system apparently took on the essential role of an extra-mitochondrial oxidizing system reoxidizing reduced NAD generated by glycolysis in lieu of mitochondrial respiration. Under these new conditions oxygen would necessarily be supplied by host hemoglobin rather than by catalase action on H_2O_2. If we accept Hoare's (1967) hypothesis that the trypanosomes evolved by successive stages into a form in which the entire life history occurs in the host (T. equiperdum, transmitted from horse to horse mechanically by coitus, is permanently dyskinetoplastic), then T. equiperdum may represent the end-point of a transition from an organism dependent on oxygen produced by catalase for consumption by the mitochondrion to one dependent on

an extra-mitochondrial GPO system devoid of catalase and driven by O_2 supplied by host hemoglobin. According to Hoare's (1967) evolutionary sequence: Crithidia → T. lewisi, congolense, vivax → T. brucei, one might predict, therefore, that the insect and culture forms of T. lewisi, T. congolense, T. vivax would exhibit intermediate amounts of catalase. The increased prominance of the peroxisomes and the diminished rotê of the mitochondrion in trypanosome evolution evidently is an irreversible parasitic specialization which forbids return to life in the insect vector.

Understanding of this fascinating replacement -- or displacement -- will only come from study of the comparative biochemistry of microbodies in trypanosomes and leishmanias. A survey of the peroxisomes of Trypanosomatidae should help clarify their role not only in trypanosomes but in other protozoan parasites which contain organelles housing para-mitochondrial respiratory systems such as in the Trichomonadinae (Muller, 1972).

APPENDIX

Buffers & Reagents

1) Ribosomal Suspension Medium (RSM buffer) pH 7.6

sucrose	250 mM
Tris	0.35 mM
KCl	0.25 mM
$MgCl_2$	0.10 mM
β-mercaptoethanol	6.5 mM
glycerol	1% v/v

in distilled water.

2) Sucrose Buffer pH 7.6

sucrose	250 mM
ethylenediaminetetraacetic acid (EDTA)	0.1 mM
Tris	1.0 mM

in distilled water.

3) DAB (Diaminobenzidine) Oxidation Medium pH 9

2-amino,2-methyl, 1,3 propanediol (Sigma) 0.05 M	10 ml
hydrogen peroxide (Baker) 1% freshly diluted from a 30% solution	0.2 ml
3,3' diaminobenzidine tetrahydrochloride (DAB, Sigma)	20 mg

adjusted to pH 9.

4) DAB Oxidation Medium pH 6

sodium acetate-acetic acid buffer	0.05 M at pH 5	10.0 ml
hydrogen peroxide (Baker) 0.1% freshly diluted from a 30% solution		0.1 ml
DAB		20 mg
manganous chloride	0.05 M	0.1 ml

adjusted to pH 6.

All DAB media were modified by the addition of 0.25 M sucrose containing 1 mg BSA/ml final concentration.

5] Calcium Formal Fixative

Anhydrous $CaCl_2$	1 gm
40% formaldehyde	10 ml
H_2O	to 100 ml

adjusted pH to 7.0→7.2 with 1N NaOH.

Crithidia fasc. Defined Medium[a]

Compound	Amt./100 ml final medium	Compound	Amt./100 ml final medium
K_3citrate•H_2O	0.1 g	L-Threonine	0.01 g
Citric acid (anhydrous)	0.045 g	L-Tryptophan	0.01 g
L-malic acid	0.02 g	L-Tyrosine ethyl ester•HCl	0.01 g
Succinic acid	0.1 g	L-Valine	0.015 g
$MgCO_3$	0.1 g	Nicotinamide	0.05 mg
Trace elements[b]	0.01 g	Ca panthothenate	0.03 mg
$Fe(NH_4)_2(SO_4)_2H_2O$	1.0 mg	NaRiboflavin PO_4•$2H_2O$	0.05 mg
CaSuccinate•H_2O	3.0 mg	Pyridoxamine•2HCl	0.006 mg
Adenosine	2.5 mg	Biotin	0.2 µg
L-Arginine•HCl	0.05 g	Thiamine HCl	0.03 mg
L-Histidine•HCl	0.01 g	Folic acid	0.03 mg
L-Isoleucine	0.02 g	Sorbitol	1.5 g
L-Leucine	0.03 g	Na_2DL-glycerophosphate•$5H_2O$	
L-Lysine•HCl	0.02 g	(25% α 75% β)	1.0 g
L-Methionine	0.015 g	Hemin[c] (in 50% TEA[d])	1.5 mg
L-Phenylalanine	0.015 g		

[a]Modified from Bacchi et al. (1969)

[b]To yield (mg/100 ml):

Fe, 0.6; Mn, 0.5; Zn, 0.5; Mo, 0.2; Cu, 0.04; V, 0.02; Co, 0.01; B, 0.01; Ni, 0.01; Cr, 0.01.

[c]Type III equine (~98%), Sigma Chemical Co.

[d]TEA, Triethanolamine (Sigma).

Computations: Regression Equations for Least Squares Fit

1) $y = a + bx$ 2) $b = \dfrac{\Sigma xy - \dfrac{(\Sigma x)(\Sigma y)}{n}}{\Sigma x^2 - \dfrac{(\Sigma x)^2}{n}}$ 3) $a = \dfrac{\Sigma (x)}{n} - \dfrac{b(\Sigma y)}{n}$

Control		Mutant	
Σx =	38,633	Σx =	24,615
Σy =	902	Σy =	1,191
Σx^2 =	36,969,811	Σx^2 =	34,907,341
$\Sigma x \cdot y$ =	855,312	$\Sigma x \cdot y$ =	1,146,518
$(\Sigma x)^2$ =	1,492,508,689	$(\Sigma x)^2$ =	512,000,000
n =	46	n =	27

by substitution,

$y = 17.9 + .002x$	$y = 3.99 + .044x$
$b = \dfrac{9,777}{4,523,970} = .002$	$b = \dfrac{108,651}{2,466,669} = .044$
$a = 17.9$	$a = 3.99$

Test For Significance of Least Squares Fit

	Control	Mutant
R =	+.428	+.340
s_x^2 =	100,532	97,344
s_x =	317	312
s_y^2 =	256	497.2
s_y =	16	22
$s_{y\,x}^2$ =	213	457.12
$s_{y\,x}$ =	$\pm$14.9	$\pm$21.3
s_b^2 =	.000047	.00018
s_b =	.00685	.0134
$\frac{b}{s_b}$ =	0.29	3.279

$\frac{b}{s_b}$ is significant at .05 level (t_{n-2}) if it is greater than 2 on the basis of a null hypothesis.

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[a]The format used is that prescribed by the editors of the Journal of Protozoology.

ERRATUM

The following citation was inadvertently omitted from the list of references:

Okabayashi, T., Ide, M., Yoshimoto, A. & Otsubo, M. 1965. Mutagenic activity of 4-nitroquinoline 1-oxide and 4-hydroxyaminoquinoline 1-oxide on bacteria. Chem. Pharm. Bull. 13, 610-1.