EFFECT OF ACRIFLAVIN ON THE KINETOPLAST OF *LEISHMANIA TARENTOLAE*

Mode of Action and Physiological Correlates of the Loss of Kinetoplast DNA

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ABSTRACT

The loss of kinetoplast DNA in *Leishmania tarentolae*, which occurs in the presence of low concentrations of acriflavin, was found to be a result of selective inhibition of replication of this DNA. Nuclear DNA synthesis was relatively unaffected and cell and kinetoplast division proceeded normally for several generations. An approximately equal distribution of parental kinetoplast DNA between daughter kinetoplasts resulted in a decrease in the average amount of DNA per kinetoplast. The final disappearance of the stainable kinetoplast DNA occurred at a cell division in which all the remaining visible kinetoplast DNA was retained by one of the daughter cells. The selective inhibition of kinetoplast DNA synthesis was caused by a selective localization of acriflavin in the kinetoplast. The apparent intracellular localization of dye and the extent of uptake at a low dye concentration could be manipulated, respectively, by varying the hemin (or protoporphyrin IX) concentration in the medium and by adding red blood cell extract (or hemoglobin). Hemin and protoporphyrin IX were found to form a complex with acriflavin. During growth in acriflavin, cells exhibited an increasing impairment of colony-forming ability and rate of respiration. No change in the electrophoretic pattern of total cell soluble proteins was apparent. The data fit the working hypothesis that the loss of kinetoplast DNA leads to a respiratory defect which then leads to a decrease in biosynthetic reactions and eventual cell death. A possible use of the selective localization of acriflavin in the kinetoplast to photooxidize selectively the kinetoplast DNA is suggested.

INTRODUCTION

The kinetoplast is a specialized portion of a mitochondrion containing a large amount of DNA. This organelle is characteristic of flagellates of the order kinetoplastida (Honigsberg et al., 1964), and seems to be especially adapted for the complex life cycles of these mostly parasitic cells (Vickerman, 1962; Rudzinska et al., 1964). The historical "kinetoplast" (Rabinowitsch and Kempner, 1899) was a small granule located at the base of the flagellum. It stained dark with dyes, such as Giemsa's, and was Feulgen-positive (Bresslau and Scremin, 1924). Numerous electron microscope and histochemical studies have since shown this granule to be a specialized portion of a highly extended, convoluted mitochondrion (Clark and Wallace, 1960; Ris, 1962; Rudzinska et al., 1964; Schulz and MacClure, 1961; Steinert, 1960, 1964; Trager and Rudzinska, 1964; Anderson and

Ellis, 1965; Vickerman, 1962). The staining properties result from the existence of a fibrous bundle of DNA situated anteriorly within the mitochondrial matrix directly opposite the basal body of the flagellum. The DNA-containing portion of the chondriome characteristically is a lightly concave, elongated disc-shaped structure which has typical mitochondrial cristae toward the posterior portion and mitochondrial tubules extending from its lateral ends. It is likely that in all the hemoflagellates (family, Trypanosomatidae) the entire mitochondrial complement of the cell is connected to the disc-shaped body containing the stainable DNA. This portion of the chondriome is a permanent fixture of the cell, whereas the mitochondrial ramifications are variable in both extent and appearance (Vickerman, 1962). The term kinetoplast will be retained to distinguish the disc-shaped structure, even though it is not a separate organelle.

The kinetoplast DNA differs from the nuclear DNA in buoyant density in CsCl (du Buy et al., 1965; Riou et al., 1966), and in annealing behavior (du Buy et al., 1966). Steinert and Steinert (1962) found that it replicates within the nuclear S period, unlike all other mitochondrial DNA which have been studied carefully (Evans, 1966; Parsons, 1965; Neubert et al., 1965). The in vivo molecular weight of the kinetoplast DNA is unknown.

One of the most interesting and useful properties of the kinetoplast is its susceptibility to elimination by certain dyes (Werbitzki, 1910). Trager and Rudzinska (1964) and Mühlpfordt (1963) found by electron microscopy that dye-induced so-called "akinetoplastic" cells lack the kinetoplast DNA structure but retain the kinetoplast membranes. There is no change in the fine structure of the nucleus or of other cytoplasmic organelles. However, the mitochondrial cristae appear degenerate. Trager and Rudzinska proposed the use of the term "dyskinetoplastic" rather than the previous akinetoplastic, because of the retention of the mitochondrial membranes.

The existence of such dye-induced "mutants" provides a convenient tool for study of the possible genetic role of the kinetoplast DNA. However, the production of dyskinetoplastic cells is not a repeatable, controllable process. No one system has been worked out in terms of cultural conditions, repeatability, and mechanism of action of the dye. This paper provides such a study. Fairly optimal experimental conditions for the production of dyskinetoplastic *Leishmania tarentolae* cells by acriflavin were obtained, and the mode of action of acriflavin was investigated.

MATERIALS AND METHODS

Materials

Neutral acriflavin was purchased from Nutritional Biochemicals Corporation (Cleveland, O.); acridine orange was purchased from Allied Chemical Corp. (National Analine Division, New York). Ilford L-4 emulsion was purchased from Ilford Chemical Company, England. Recrystallized hemin was obtained from the Nutritional Biochemicals Corporation, protoporphyrin IX from Calbiochem. (Los Angeles, Calif), and recrystallized horse hemoglobin from Pentex, Inc., Kankakee, Ill.

Thymidine-^{3}H (specific activity 6.7 c/mmole) was purchased from the New England Nuclear Corp. (Boston, Mass.) and Tergitol TP-9 from Union Carbide Corporation (Chemical Division, New York).

Media

Red blood cell extract was prepared from horse red blood cells washed once with Locke's solution. The cells were resuspended in 2 volumes of Locke's solution and were frozen and thawed three times in ethanol-dry ice. Stroma were removed by centrifuging at 18,000 *g* for 30 min at 4°C. The extract was stored frozen at −20°C in small quantities. The standard addition to medium C (Trager, 1957) involved a 1/30 dilution which gave a final hemoglobin concentration of 3.6 mg/ml. The measured hemoglobin heme was 110 μg/ml (Neufeld et al., 1958).

Hemin was dissolved as the hematin molecule in 0.05 N NaOH at a concentration of 2 mg/ml. It was sterilized by filtration through Millipore HA filter and stored frozen in small quantities. Unless otherwise noted, hemin was standardly added at a concentration of 24 μg/ml to medium C just prior to inoculation.

The standard medium to wash cells (SBG) consisted of 0.15 M NaCl–0.02 M glucose–0.02 M phosphate buffer (pH 7.9).

Acriflavin was dissolved in redistilled water at 1 mg/ml, sterilized by autoclaving, and kept in darkness at 4°C. The same bottle of dye was used for all experiments.

Cells

A strain of *Leishmania tarentolae* was used which has been growing continuously in a defined medium (medium C; Trager, 1957) since 1959. Cells were grown at 27°C in the dark in 30 ml of medium C in stationary 250-ml Erlenmeyer flasks equipped with

cotton plugs wrapped in gauze and covered with parafilm, or in 300-ml quantities in 2500-ml flat-bottomed flasks. No antibiotics were used. Cultures were inoculated with about 0.5–1.0 × 10^6 cells per milliliter. Cell counts were made by diluting a sample 1:1 with 3.7% formalin in 0.15 M NaCl–0.015 M Na citrate (pH 7.0), and counting 300–600 cells in a hemacytometer at × 480.

Before being stained with Giemsa's, cells were mixed with an equal volume of 1% albumin in 0.25 M sucrose, centrifuged down at 2,500 rpm for 10 min (International PR-2 refrigerated centrifuge), and smeared. Smears were rapidly air-dried, fixed with absolute methanol for 5 min, and stained with Giemsa's for 30 min.

CsCl Gradient Centrifugation

This procedure was carried out according to Meselson et al. (1957). The solutions were centrifuged in a Spinco model E at 44770 rpm for at least 20 hr. Tracings of the UV absorption negatives were obtained with a Joyce-Loebell densitometer.

Isolation of DNA

The phenol-pH 9 method of Saito and Miura (1963) was used. If little material was at hand, the aqueous layer of the phenol extract was extracted 10 times with ether and dialyzed for 3 days against four changes of 0.15 M NaCl at 4°C. It was then treated with 10 μg pancreatic ribonuclease per milliliter for 30 min at 37°C and quickly dialyzed again. DNA concentration was measured by the Burton (1956) modification of the diphenylamine assay, with calf thymus DNA as a standard.

Fluorescence Microscopy

A Zeiss fluorescence microscope was used with excitor filters BG-3 and BG-12 and barrier filters 50–47.

Radioautography

The cells were smeared onto presubbed slides (Caro, 1964), air-dried, and fixed in methanol. Enzyme digestion was performed at this point if desired: deoxyribonuclease, 0.02% in 0.02 M phosphate buffer, 3 mM $MgSO_4$ (pH 6.8) 2 hr at 24°C. The slides were then extracted with cold 5% trichloroacetic acid for 5–10 min, given four 30-min washes in 70% ethanol and a 5 min wash in 95% ethanol, and air-dried. They were dipped in 1:1 diluted Ilford L-4 emulsion, dried, and stored in light-proof boxes at 4°C. Exposed slides were developed with Kodak D-19 developer (Eastman Kodak Co., Rochester, N. Y.). If necessary, they were stained with Giemsa's according to Gude et al. (1955).

Visible Irradiation of L. Tarentolae

Cells were treated with acriflavin for 3–5 hr at 27°C in medium C and then washed three times in

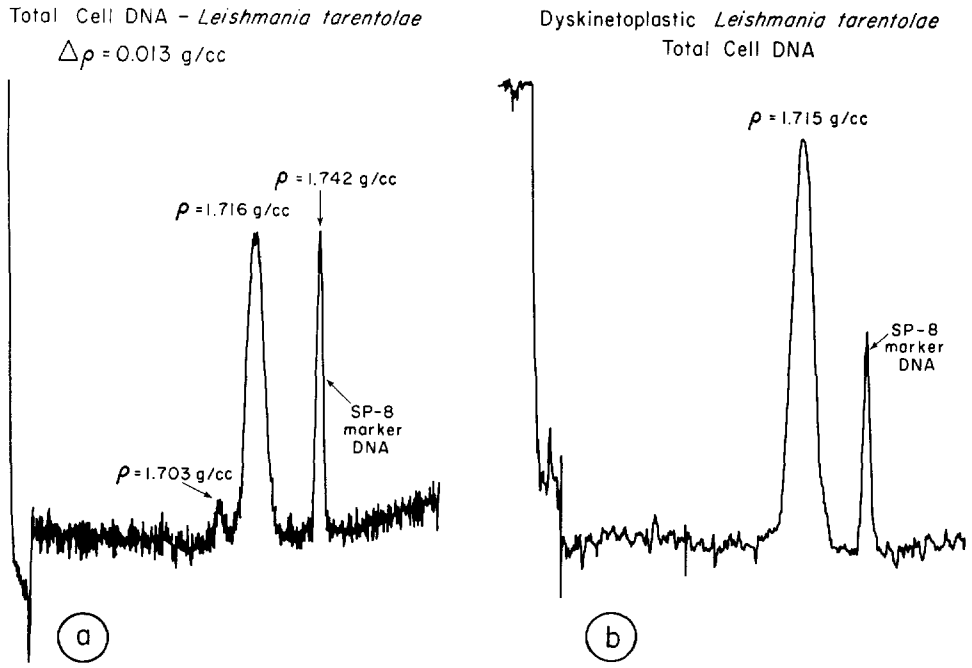

FIGURE 1 CsCl analytical ultracentrifugation of total cell DNA from normal (*a*) and 70% dyskinetoplastic (*b*) *L. tarentolae*. Phage SP-8 DNA was used as a density marker.

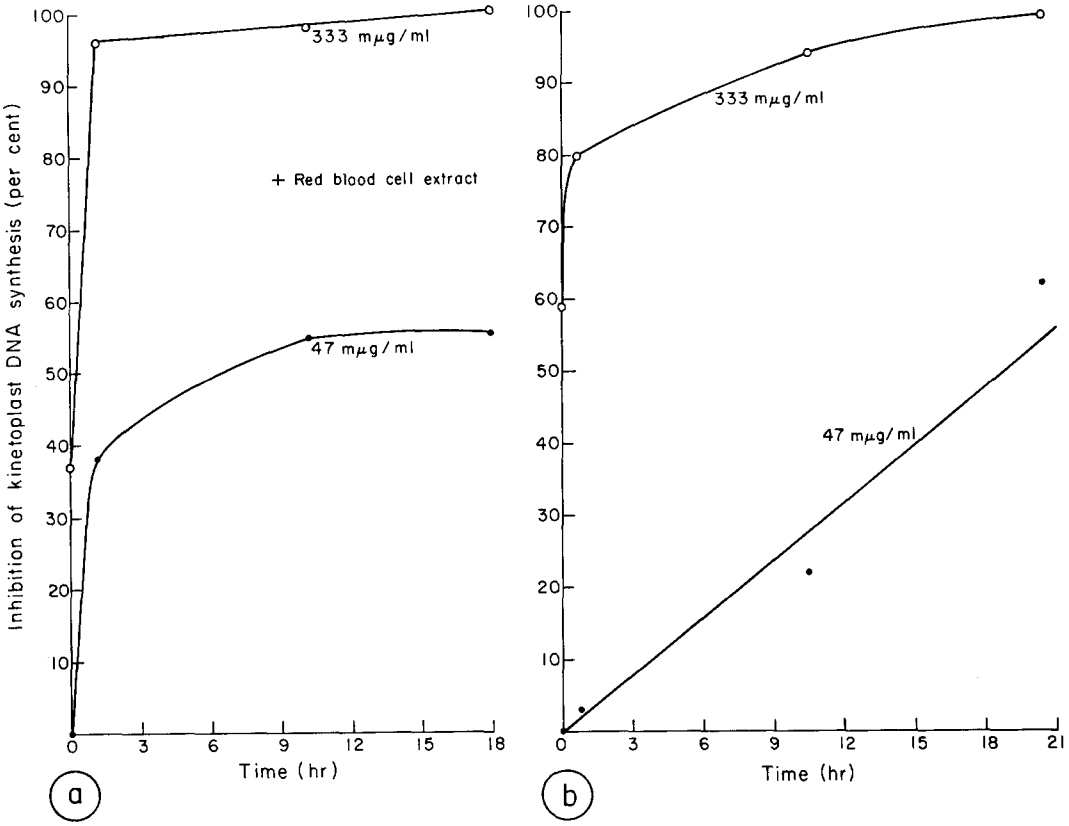

FIGURE 2 The inhibition of kinetoplast DNA synthesis by acriflavin. Cells were in medium C with (*a*) and without (*b*) red blood cell extract. Dye was added at 0 hr, samples of the cultures were centrifuged, and the pellets were resuspended in 0.2 ml medium C, 1% bovine serum albumin. These samples were then given 30-min pulses of thymidine-^{3}H (50 μc/ml; 6.7 c/mmoles) at the indicated times, smeared, and processed for radioautography. Dye-treated cells were compared to untreated controls at each point. The following method was used to calculate per cent inhibition of kinetoplast DNA synthesis: (N, nuclear label; K, kinetoplastic label; c, control; and E, experimental):

$$\frac{\%(N + K)_c + \%K_c] - [\%(N + K)_E + \%K_E]}{[\%(N + K)_c + \%K_c]}$$

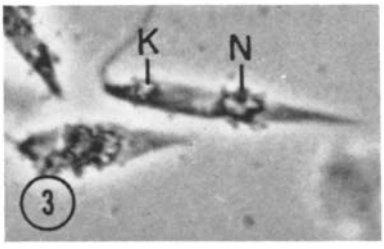

FIGURE 3 Radioautograph of incorporation of thymidine-^{3}H into nuclear (N) and kinetoplast (K) DNA of *L. tarentolae*. Grown for six generations in presence of label. 1 month exposure. Phase-contrast; × 1000.

SBG. The cells were irradiated in 2-ml portions in Pyrex tubes (1.3 cm ID) and stirred with a small magnetic bar. The light source was a water-filtered xenon arc lamp (Bausch & Lomb Incorporated, Rochester, N. Y.), with an intensity of approximately 0.68 × 10^6 erg/cm^2/sec.[1]

Agar Plating

The cells were plated by spreading 0.1–0.2 ml of cells in SBG–1% bovine serum albumin evenly over the agar surface by tilting. The plates were taped,

[1] I thank Mr. Paul Rosen for carrying out this measurement.

inverted, and stored at 27°C. The agar medium consisted of 1% Bactoagar (Difco Laboratories, Detroit, Mich.) and 2.5% rabbit blood.

Electrophoresis and Protein Assay

The Lowry et al. (1951) Folin-phenol assay was employed with bovine serum albumin as the standard.

Electrophoresis was carried out on 1 × 6¾ inch strips of Sepraphore III (Gelman Instrument Corporation) at 450 v for 45 min at 4°C in high resolution buffer, pH 8.6, (Gelman Instrument Company, Ann Arbor, Mich.) diluted in proportions of one packet per 2200 ml of water. Strips were stained with Ponceau S in 5% trichloroacetic acid and destained in acetic acid.

RESULTS

Mode of Action of Acriflavin

LOSS OF THE SATELLITE BAND: Total cell DNA isolated from normal *L. tarantolae* gave two bands on CsCl equilibrium gradient centrifugation[2] (Fig. 1 a), the satellite being lighter in buoyant density and hence higher in per cent adenine plus thymine if no unusual bases were present (Schildkraut et al., 1962). The satellite band, which represented 5–10% of the total cell DNA in several CsCl gradients, could not be found in total cell DNA from 70% dyskinetoplastic *L. tarentolae* (Fig. 1 *b*). This indicates that the satellite is probably kinetoplast DNA. However, by use of another method, light microscope radioautography of cells labeled over six generations, the relative amount of kinetoplast DNA in normal cells was found to be 21%. The most probable explanation for the failure to observe the expected value of kinetoplast DNA in the phenol-extracted preparation is a differential dissociability of the two types of DNA from their respective proteins and a loss of the protein-bound kinetoplast DNA in the interface.

The sedimentation rate of the nuclear DNA, measured by velocity sedimentation in sucrose gradients, was relatively unaffected by the acriflavin treatment.

INHIBITION OF KINETOPLAST DNA REPLICATION: A rapid inhibition of the synthesis of kinetoplast DNA was brought about by treatment of cells with acriflavin (Fig. 2). Concentrated samples of cells were given 30-min pulses of thymidine-^{3}H in medium C containing 1% bovine serum albumin and then were smeared and processed for radioautography. The acid-insoluble label was demonstrated to be in DNA by the use of DNase digestion of control slides. The kinetoplast grains could easily be distinguished from the nuclear grains, as illustrated by the overexposed radioautograph in Fig. 3.

Two acriflavin concentrations were examined: 47 and 333 mμg/ml. It will be shown later that the optimal level for the production of dyskinetoplastic cells under the proper cultural conditions is approximately 150–200 mμg/ml. As seen in Fig. 2 *a*, the inhibition of kinetoplast DNA synthesis in medium C with red blood cell extract rapidly reached a maximum of 50–60% with 47 mμg/ml and 90–100% with 333 mμg/ml. The actual data are given in Table I.

The absence of red blood cell extract in the culture medium (medium C) decreased the rate and extent of inhibition of kinetoplast DNA synthesis (Fig. 2 *b*). The use of red cell extract derived from Trager's observation (Personal communica-

TABLE I

Effect of Acriflavin on DNA Synthesis in L. tarentolae

Acriflavin	Time	Cells labeled	Distribution of label		
			$N + K$	N	K
mμg/ml	*hr*	%	%	%	%
0	0	16	81	18	1
	1.3	23	83	13	4
	10.5	35	92	4	4
	18	31	92	7	1
47	0	14	80	17	3
	1.3	21	52	46	2
	10.5	36	41	57	2
	18	34	40	58	2
333	0	16	50	48	2
	1.3	26	3	97	0
	10.5	27	2	98	0
	18	19	0	100	0

The data for Fig. 2 *a*. Cells were grown in medium C with red blood cell extract and hemin at 20 μg/ml, pulsed with thymidine-^{3}H for 30 min at the indicated times, and processed for radioautography. The percentages of cells having both nuclear and kinetoplast label ($N + K$), pure nuclear label (N), and pure kinetoplast label (K) are given. An organelle was scored as labeled if it had more than two grains. 200–500 cells were counted for each point.

[2] I would like to thank Dr. David Luck for assistance with the analytical ultracentrifugation.

tion.) that the production of dyskinetoplastic cells was greatly stimulated by such an addition. Human, horse, and rabbit red blood cell extracts and horse hemoglobin, all were equally effective.

The increase in the percentage of cells undergoing total DNA synthesis (cells with more than two grains) with time, at the various acriflavin levels, is shown in Fig. 4. The higher acriflavin concentration clearly affected the rate of total cell DNA synthesis more than the lower concentration. It was also demonstrated by direct counting experiments that low acriflavin concentrations had no great effect on total cell uptake of thymidine-^{14}C over a 2 hr period. These facts indicate that the lower acriflavin level, which inhibits kinetoplast DNA synthesis by 50–60%, has no great effect on nuclear DNA synthesis, whereas the higher level significantly affects nuclear DNA synthesis also. The failure (in Fig. 4) to observe a significant difference in the presence (*a*) or absence (*b*) of red blood cell extract is probably due to the procedure of scoring a cell as undergoing DNA synthesis if it has more than two grains over the nucleus or kinetoplast. The inhibition of kinetoplast DNA synthesis would not be apparent in this method.

It should be noted that all dye concentrations, unless otherwise indicated, were prepared on a dry weight basis with commercial "neutral acriflavin" (Nutritional Biochemical Corporation). Purified acriflavin gave identical results at lower dye concentrations.

KINETICS OF LOSS OF STAINABLE KINETOPLAST DNA: The increase in the percentage of dyskinetoplastic cells as a function of time of growth in a fairly optimal level of acriflavin was measured (Fig. 5). Cells were scored from Giemsa-stained smears and only those cells having nothing at all visible in the kinetoplast region were termed dyskinetoplastic. Cells with small dots for the kinetoplast were termed "normals," although they are clearly abnormal in their content of kinetoplast DNA. Since the staining properties of the kinetoplast are due mainly, if not entirely, to the

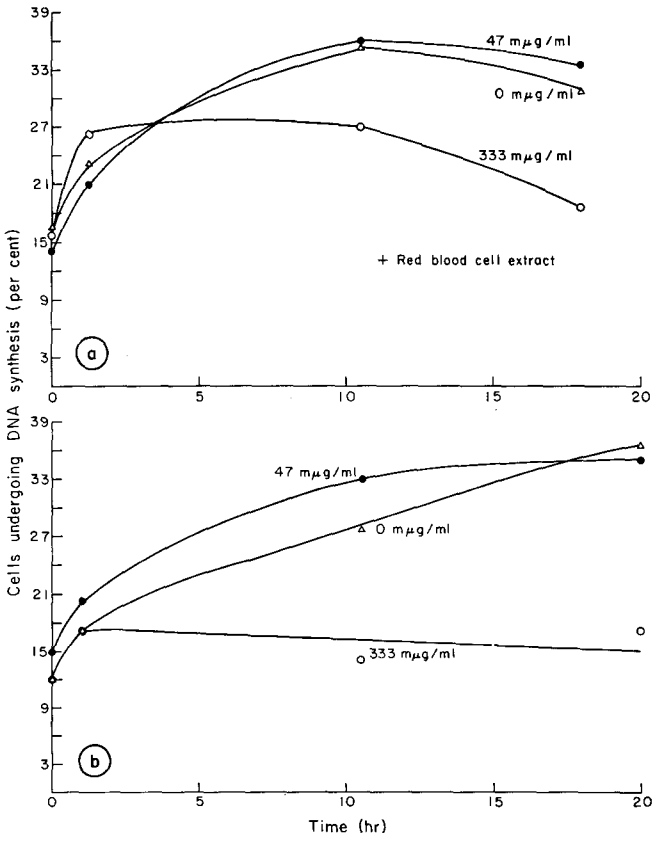

FIGURE 4 The effect of acriflavin on the percentage of cells undergoing total DNA synthesis. See Fig. 2 for details. Cells with more than two grains were scored as undergoing DNA synthesis.

kinetoplast DNA (Steinert, 1965), the size of the kinetoplast granule is a valid indication of the amount of DNA. The loss of the ability of the kinetoplast to stain with Giemsa's after DNase treatment of fixed smears was confirmed for *L. tarentolae*. The complete loss of acridine-orange staining of the kinetoplast region of dyskinetoplastic cells was also confirmed for *L. tarentolae*.

Fig. 5 also presents the growth curves of the untreated and acriflavin-treated cells. The log-phase growth rates are indentical (9 hr doubling time), but there is some inhibition of the final cell yield under these cultural conditions. The curve for the percentage of dyskinetoplastic cells shows a lag for about 20 hr, then a sharp rise and a plateau. When plotted against the number of cell divisions, the increase in dyskinetoplastic cells is linear after three cell divisions, as would be expected considering the hypothesis that the kinetoplast DNA is inhibited from replicating and is being diluted out.

MORPHOLOGICAL DISTRIBUTION OF STAINABLE KINETOPLAST DNA DURING GROWTH IN ACRIFLAVIN: For comparison with dye-treated cells, normal, untreated cells are shown in the micrograph of Fig. 6. The stainable kinetoplast (*K*) is visible as a prominent dark granule at the base of the flagellum. The other granules observed in the vicinity of the nucleus in the cells at the lower left are most probably "volutin" granules as described by Ormerod (1961).

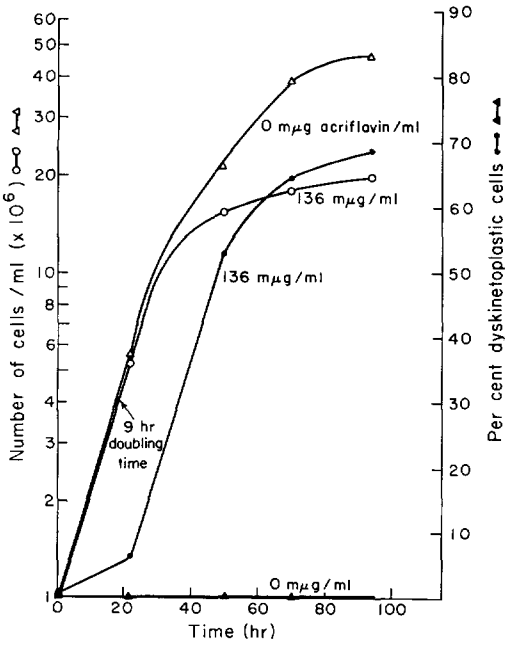

FIGURE 5 Kinetics of the effect of acriflavin on *L. tarentolae*. Comparison of the growth curves of cells with and without acriflavin at a fairly optimal level (136 mμg/ml), and the kinetics of appearance of completely dyskinetoplastic cells. Cells were in medium C, with red blood cell extract; the hemin concentration was 5 μg/ml.

The dye-treated situation is schematically represented in Fig. 7, which shows the major division forms seen in a log-phase, acriflavin-treated culture. The first few divisions in the presence of the dye produce a decrease in the size of the stainable kinetoplast, as illustrated by the micrograph in Fig. 8 *a*.

The final disappearance of the stainable kinetoplast occurs at an "all and none" division, which yields one normal and one dyskinetoplastic cell (Fig. 8 *b* and *c*). These observations would indicate that the percentage of such all and none divisions increase with time. This is verified in Fig. 9.

The transverse nature of the all and none divisions presented in Fig. 8 *b* and *c* and the longitudinal nature of the normal divisions in Fig. 8 *a* are purely fortuitous and merely represent different times in the division process.

Completely dyskinetoplastic cells can continue to divide at least once. This is demonstrated by the data in Table II in which are presented the percentages of binucleate division forms of normal and dyskinetoplastic cells during growth in acriflavin. Binucleate division forms of dyskinetoplastic cells occur at about one-half the rate of normal cells. Such forms are shown in the micrographs of Fig. 8 *d*, *e*, and *f*. Table III *a* gives the actual percentages of each of the major division forms seen in slides prepared at various times during growth in acriflavin.

Table III *b* demonstrates that the total number of cells having some kinetoplast DNA increases approximately logarithmically for two to three cell divisions and then remains constant, as would be implied by a failure to transfer replicates of kinetoplast DNA into both daughter cells.

ACRIFLAVIN DOSE-RESPONSE CURVES, COMMERCIAL DYE: The variation in final cell yield and percentage of dyskinetoplastic cells with the acriflavin concentration is shown in Fig. 10. The cells were grown to stationary phase in medium C with (*a*) and without (*b*) red blood cell extract. The hemin concentration was 5 μ/ml. In both cases there is an increasing inhibition of cell growth

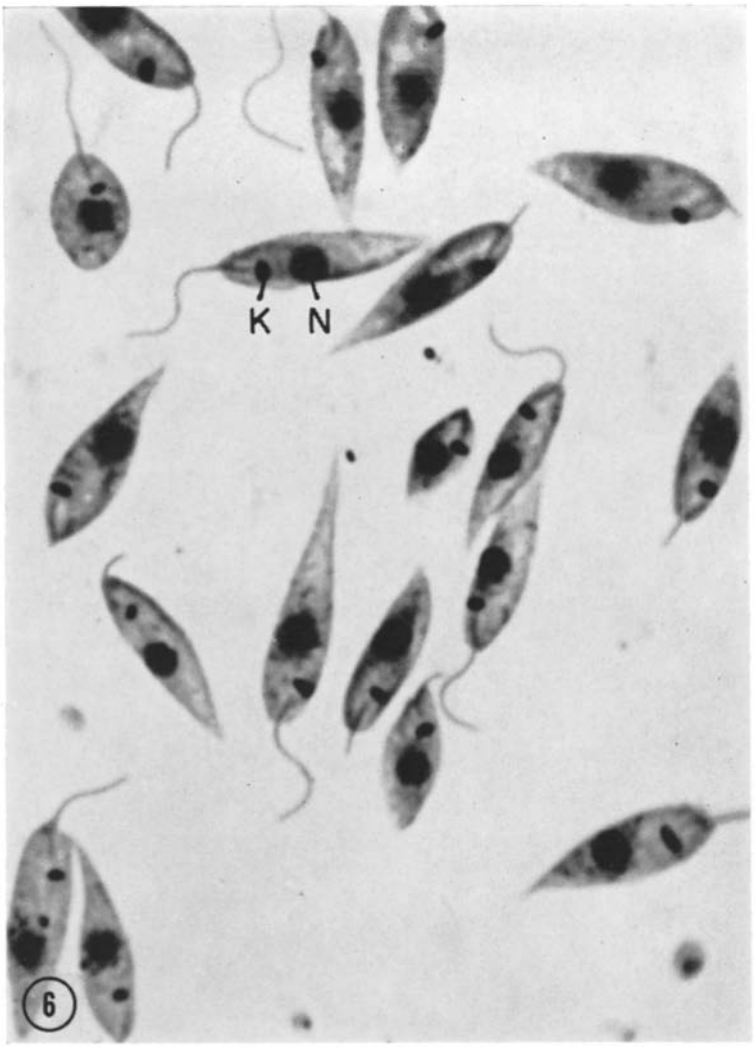

FIGURE 6 Normal, untreated *L. tarentolae* grown in medium C. Giemsa stain. The stainable kinetoplast (*K*) and the nucleus (*N*) are apparent. × 1300.

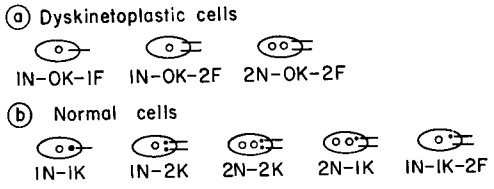

FIGURE 7 Schematic diagram of major division forms seen in Giemsa-stained smears of cells growing in presence of acriflavin (136 mμg/ml). *N*, nucleus; *K*, kinetoplast; *F*, flagellum.

with increasing concentration of dye. The percentage of dyskinetoplastic cells reaches a maximum at about 50% growth inhibition and then drops or reaches a plateau level.

The presence of red blood cell extract (Fig. 10 *a*) increases the maximum percentage of dyskinetoplastic cells obtained but does not greatly affect the total cell yields under these cultural conditions.

An identical experiment at a high hemin concentration (20 μg/ml) is shown in Fig. 11. In this case the presence of red cell extract both increases

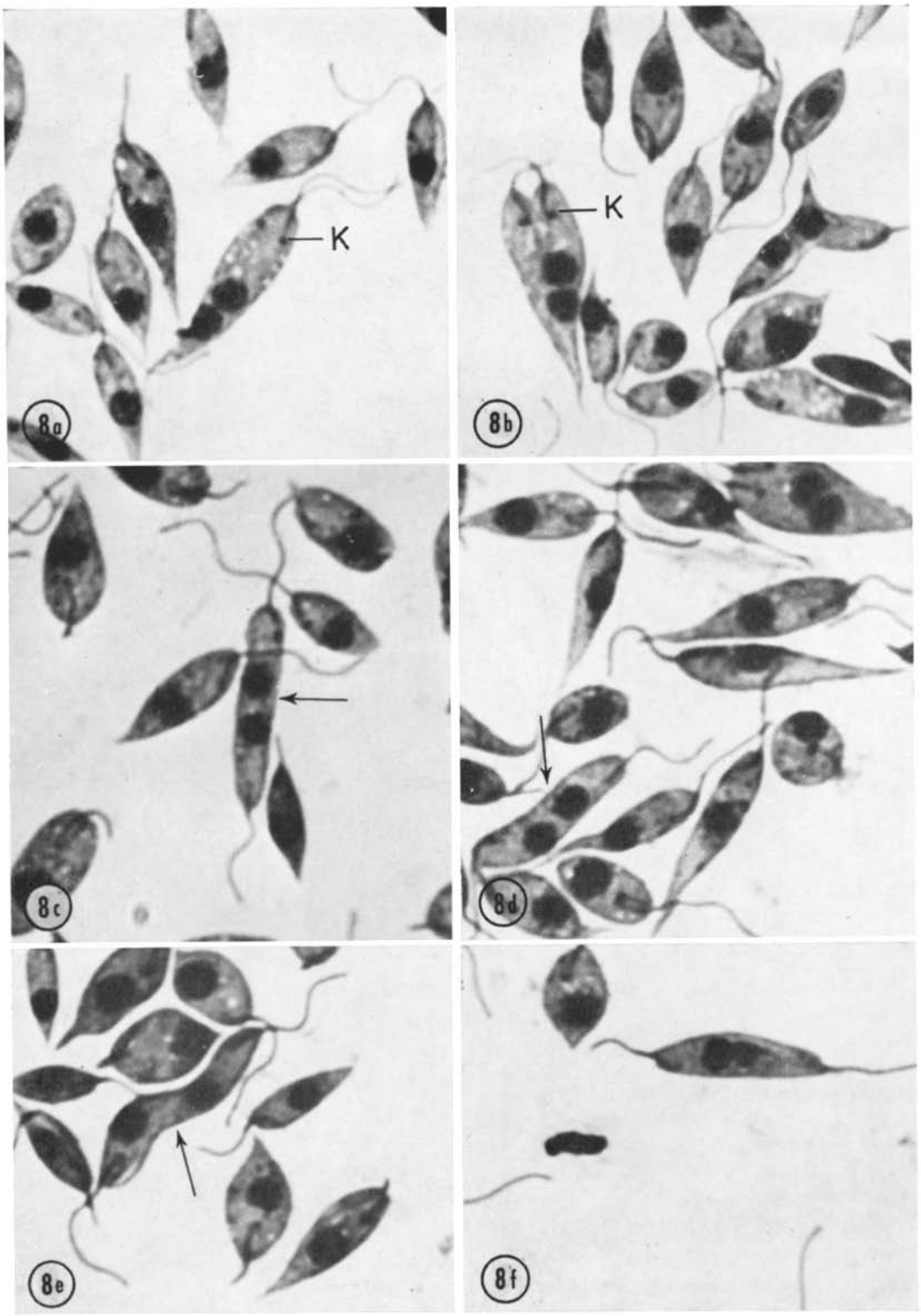

FIGURE 8 Abberrant division forms seen in acriflavin-treated (136 mμ/ml) culture. *a* and *b*, Decrease in size of stainable kinetoplast (*K*) upon cell division. *c*, all and none kinetoplast division (arrow). *d–f*, Division of dyskinetoplastic cells (arrows). Giesma stain. × 1300.

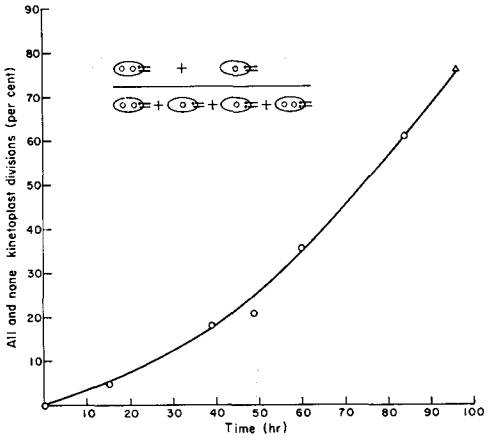

FIGURE 9 The increase in the percentage of all and none division forms during growth in presence of acriflavin (136 mμg/ml).

the maximum percentage of dyskinetoplastic cells obtained and causes a greater inhibition of cell growth at lower dye concentrations. The data from this experiment are replotted in Fig. 12 as the percentage of inhibition of cell growth, so that the effect of red blood cell extract in increasing the sensitivity of the cells to the dye can be seen more clearly.

ACRIFLAVIN DOSE-RESPONSE CURVES-PURIFIED DYE:[3] Acriflavin (2,8 diamino-N-methylacridinium chloride) was purified from commercial "neutral acriflavin" by the method of Albert (1966). Purity was ascertained by paper chromatography on Whatman No. 1 paper in butanol-5-*N*-acetic acid (7:3) (Albert, 1966, page 150). Purified acriflavin gave an R_f of 0.54 and proflavin 0.63. The absorption spectrum of the purified sample in phosphate buffer (pH 6.8, $\Gamma/2 = 0.1$) was identical with that reported by Tubbs et al., (1964) for purified acriflavin. Commercial acriflavin separated into two components on chromatography, corresponding to proflavin and acriflavin. The molar extinction coefficient of 4.68×10^4 (452 mμ) of Tubbs et al. (1964) was used for calculation of concentrations of the purified acriflavin.

A comparison of dose-response curves of cells treated with commercial acriflavin (*a*) and purified acriflavin (*b*) is shown in Fig. 13. The morphological response to purified acriflavin is identical with the response to the commercial dye discussed above. However, the optimal concentration of purified acriflavin (*b*), in terms of percentage of dyskinetoplastic cells, is 60–90 mμg/ml, whereas the optimal concentration of commercial dye is about 200 mμg/ml (*a*). Also the initial slope of the curve for per cent dyskinetoplastic cells with purified dye is approximately twice that of the curve with commercial dye. This implies that the impurities in the commercial dye are inactive in producing dyskinetoplastic cells and possibly even inhibitory. This was verified by repeating the above experiment with proflavin (dihydrochloride; Allied Chemical Corp.), the main impurity in commercial acriflavin. Fig. 14 demonstrates that proflavin is entirely inactive in both the inhibition of cell division and the production of dyskinetoplastic cells until it is in a relatively high concentration (790 mμg/ml). However, the normal amount of 20% proflavin in commercial acriflavin could not account for the large differences observed, unless the proflavin were competing with acriflavin and inhibiting its effects; but this was not investigated.

THE EFFECT OF HEMIN ON THE RESPONSE TO ACRIFLAVIN: *L. tarentolae* is a hemin-dependent organism; protoporphyrin IX (27 μg/ml) and iron could not substitute for hemin in medium C. As demonstrated above (Figs. 10 and 11), the quantitative response to acriflavin is highly dependent on the hemin concentration, especially in the presence of red cell extract. A

TABLE II

Division Forms of Dyskinetoplastic and Normal Cells from Same Culture at Various Times During Growth in Presence of Acriflavin (136 mμg/ml)

Time	Dyskinetoplastic cells	Binucleate division forms	
		Normal	Dyskinetoplastic
hr	%	%	%
15	0.7	10	—
39	16	10	4
49	31	6	3
60	44	10	3
84	62	10	4

The medium contained red blood cell extract and hemin (20 μg/ml).

[3] These experiments were performed in collaboration with Mr. Andrew Balber.

TABLE III

Percentages of Major Division Forms in Acriflavin-Treated Culture and Increase in Normal Cells During Growth of Culture

Table III *a*

		Dyskinetoplastic			Normal				
		1N-0K-1F	1N-0K-2F	2N-0K-2F	1N-1K	1N-2K	2N-2K	2N-1K	1N-1K-2F
Time	Dyskinetoplastic								
hr	%	%	%	%	%	%	%	%	%
15	0.7	—	—	—	80	10	9	1	0
39	16	94	2	4	86	4	7	2	1
49	31	91	6	3	85	8	3	3	1
60	44	93	4	3	79	6	5	5	5
84	62	95	1	4	85	6	2	8	1

Table III *b*

Time	Dyskineto-plastic	Cell concentration	No. normals
hr	%	$\times 10^6/ml$	$\times 10^6/ml$
0	0	1.0	1.0
22	7.0	5.5	5.1
51	53	15	7.4
70	65	18	6.4
95	69	20	6.3

The cells were in medium C with red blood cell extract and hemin at 20 μg/ml. The acriflavin concentration was 136 mμg/ml (commercial dye). 200–600 cells were counted for each value. Tables III *a* and *b* represent separate experiments. *N*, nucleus, *K*, kinetoplast; *F*, flagellum.

growth-dose response curve for untreated normal cells is presented in Fig. 15. Cells could be subcultured continuously at 0.2 μg/ml, although the standard hemin concentration in Trager's (1957) medium C was 24 μg/ml. Continuous growth at 0.2 μg/ml had no effect on the shape of the growth curve or on final cell yields. The actual minimal nutritional requirement for hemin is probably even lower than 0.2 μg/ml because hemin tends to form aggregates in aqueous solutions (Shack and Clark, 1947). It is of some interest that the hemin-deprived cells had long flagella, unlike the vitamin-deprived cells of Trager (1957) which were aflagellate.

The situation with dye-treated cells was quite different and is shown in Fig. 16. The percentages of dyskinetoplastic cells obtained at each hemin level are plotted on the same graphs. Protoporphyrin IX could substitute for hemin, in this effect, in approximately equimolar quantities, provided the cells were given, in addition, the minimal nutritional requirement of 0.2 μg hemin per milliliter.

It is clear from Fig. 16 *a* that increasing the hemin concentration in the presence of red blood cell extract allows more cell divisions to occur and thereby produces a greater yield of dyskinetoplastic cells. This is shown more clearly in Fig. 17, in which the data from Fig. 16 *a* are replotted in terms of percentage of dyskinetoplastic cells *versus* percentage inhibition of cell growth by the dye. Recrystallized horse hemoglobin could substitute for horse red blood cell extract in this effect.

In the absence of red cell extract (Fig. 16 *b*) the cells are affected by acriflavin only at low hemin levels (< 10 μg/ml). This experiment agrees quite well with that described previously in Fig. 10. Fig. 16 *b* also demonstrates that the action of red blood cell extract is not due to its being a supplier of hemin, in the form of hemoglobin. This was demonstrated more directly by giving to cells with low-hemin increasing concentrations of red

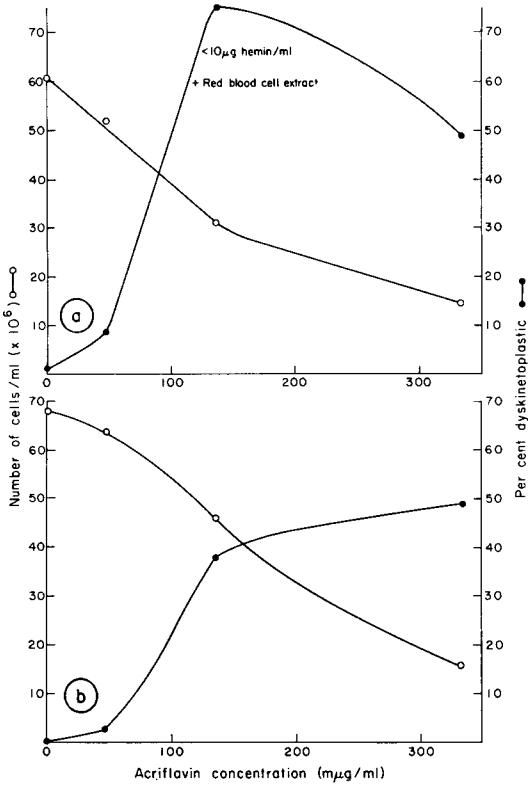

FIGURE 10 Acriflavin dose-response curve at a low hemin level (5 μg/ml). Cells grown for 4 days in medium C with (*a*) and without (*b*) red blood cell extract.

blood cell extract in the presence of acriflavin, and getting no release from growth inhibition (Table IV).

It is of some interest that dye-treated cells grown with less than 20 μg hemin per milliliter are aflagellate, unlike those grown in absence of dye. It is possible that the dye inactivates certain vitamins at low hemin concentration or competes with such vitamins (such as riboflavin, for example) for uptake into the cell, and thereby inhibits formation of flagella.

ROLE OF RED BLOOD CELL EXTRACT AND HEMIN ON UPTAKE AND LOCALIZATION OF ACRIFLAVIN IN THE CELL: Cells treated with low levels of acriflavin at high hemin levels were found by fluorescence microscopy to have an apparent exclusive kinetoplast localization of dye (Fig. 18). Cells treated with acriflavin at low hemin levels were observed to have the dye in the nucleus and cytoplasm as well. Fig. 19 is a schematic diagram of the effect of hemin and also red blood extract on the localization of acriflavin at various concentrations.

Red cell extract seems mainly to increase the

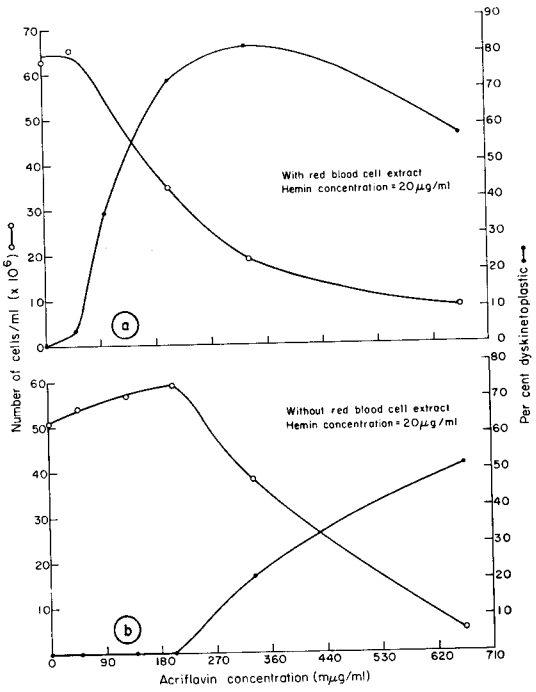

FIGURE 11 Acriflavin dose-response curve at high hemin level (20 μg/ml). Cells grown for 4 days with (*a*) and (*b*) without red cell extract.

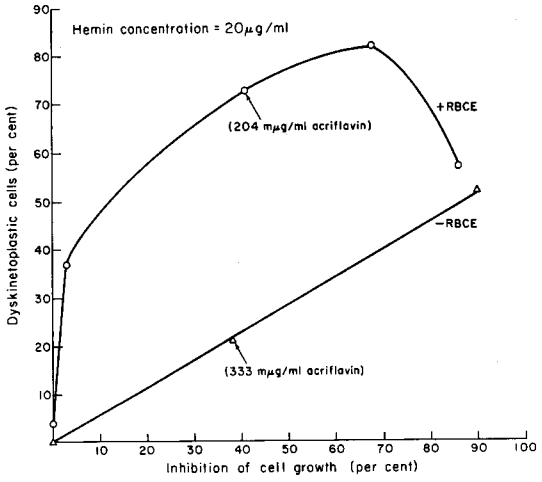

FIGURE 12 Variation of percentage of dyskinetoplastic cells with per cent inhibition of cell growth by various concentrations of acriflavin. Data from experiment in Fig. 11.

total uptake of dye by the cell and apparently does not greatly affect the intracellular distribution.

It should be noted that cells treated with the concentration of acriflavin which produces the maximal per cent yield of dyskinetoplastic cells (136 mμg/ml) have the dye mostly in the kinetoplast, but also in the nucleus and cytoplasm. This explains why the maximal percentage of dyskinetoplastic cells is produced at a growth inhibition of 50–70% (Figs. 12 and 13).

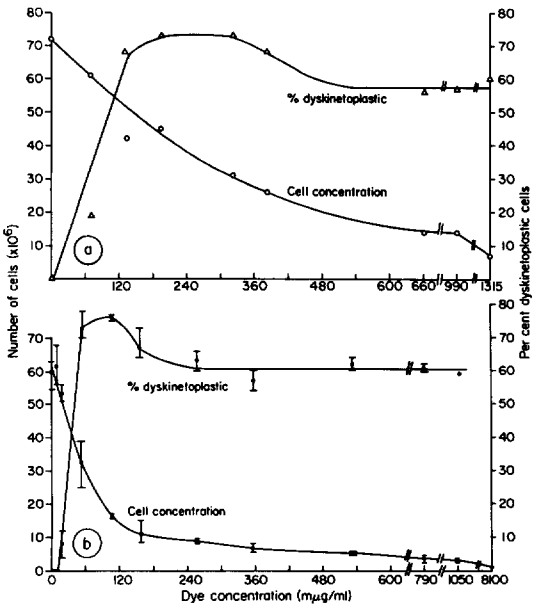

FIGURE 13 Comparison of dose-response curves of cells treated (in medium C with red blood cell extract) with commercial neutral acriflavin (*a*) and purified acriflavin (*b*). Each point in *b* is the average of three flasks with the given range.

The question arises about the mechanism involved in the ability of hemin to produce the observed effect. The possibility that hemin quenches the fluorescence directly or destroys the dye by peroxidation was examined qualitatively. It was found that the addition of 20–200 mμg hemin per milliliter to various concentrations of acriflavin (1–100 μg/ml in 0.02 M Tris-HCl, pH 7.6, or in medium C) had no discernible effect on the fluorescence of the dye as observed under low power (×100) by fluorescence microscopy. Moreover, the addition of a few drops of 30% hydrogen peroxide to 2 ml of the acriflavin-hemin solution caused no apparent diminution in the intensity of fluorescence within 10 min. Hence it is unlikely, but not completely disproven, that hemin directly quenches extrakinetoplastic dye fluorescence or destroys the dye by peroxidation.

The possibility was examined that hemin acts at the cell membrane and limits the total uptake of dye. Cells were pretreated with acriflavin (50 mμg/ml) in low-hemin medium C containing red blood cell extract, and then a high concentration of hemin (20 μg/ml) was added. Prior to the addition of the hemin the dye had been visible in the kinetoplast, nucleus, and cytoplasm. After 1 hr in the high-hemin solution there was a marked diminution of fluorescence in the cytoplasm and nucleus but no obvious effect on the kinetoplast fluorescence. These results indicate that hemin acts within the cell and either releases the dye or quenches its fluorescence. It remains an open question whether hemin also affects the relative intracellular distribution of the dye.

COMPLEX FORMATION BETWEEN ACRI-

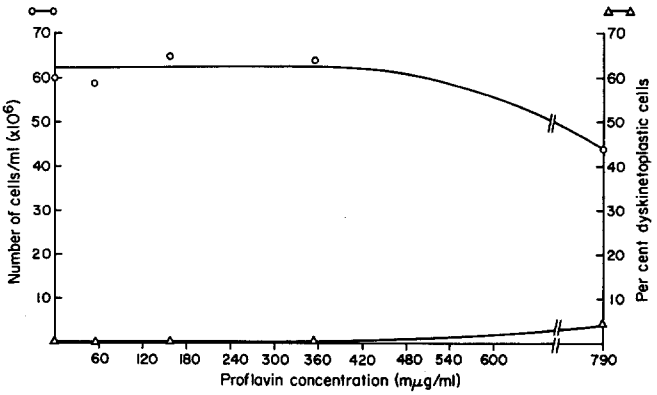

FIGURE 14 Inactivity of proflavin in the inhibition of cell growth and production of dyskinetoplastic cells. Cells were grown for 4 days in medium C with red blood cell extract and 20 μg hemin/ml.

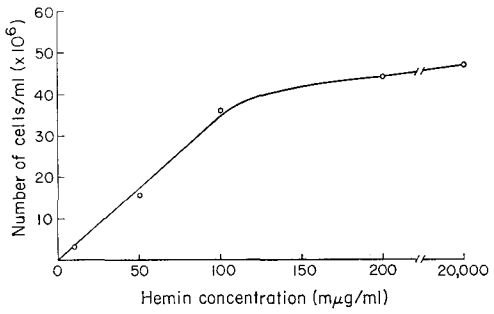

FIGURE 15 Growth response of *L. tarentolae* to increasing hemin concentrations in medium C. Cells were grown for one subculture in the carry-over amount of hemin (660 mμg/ml) and then used as inocula for this experiment. Cells were grown for 4 days.

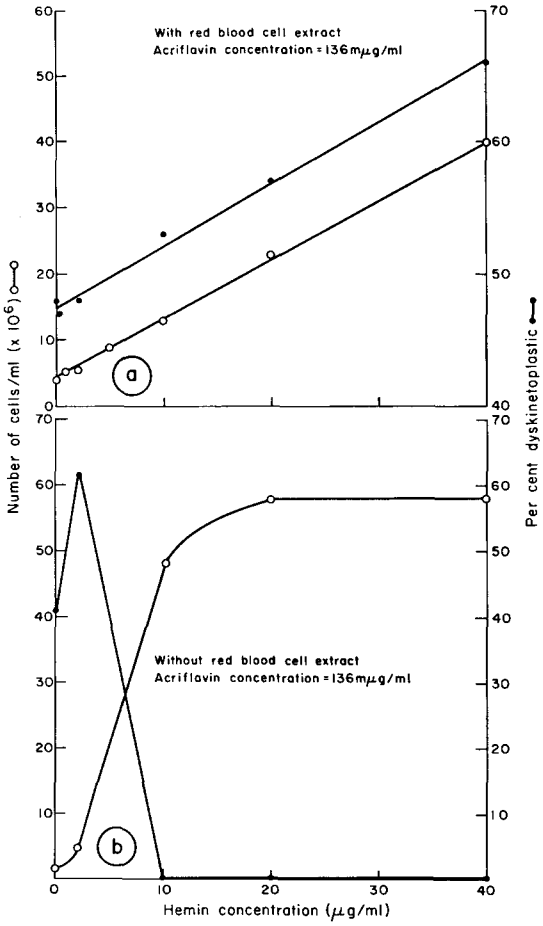

FIGURE 16 Response of acriflavin-treated cells (136 mμg/ml) to increasing hemin concentrations in medium C with (*a*) and without (*b*) red blood cell extract. Cells grown for 4 days.

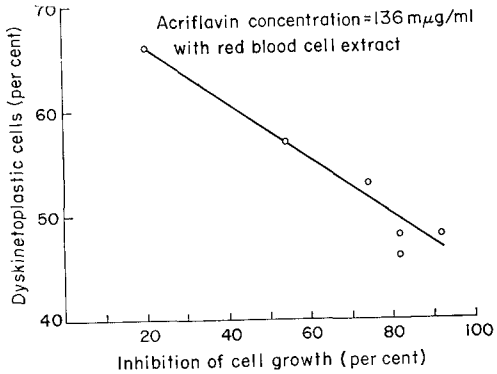

FIGURE 17 Variation in the percentage of dyskinetoplastic cells with per cent inhibition of cell growth by acriflavin (136 mμg/ml) at various hemin concentrations. Data are from experiment in Fig. 16 *a*.

TABLE IV

Effect of Increasing Concentrations of Red Blood Cell Extract on Response of L. tarentolae to Acriflavin in Low-Hemin Medium C

Hemin concentration	Red blood cell extract	No. of cells/ml	Notes
μg/ml	%	*×10⁶*	
20	3.3	23	Long flagella
0.2	3.3	4.7	Aflagellate
0.2	6.7	4.0	"
0.2	16.6	5.1	"
0.2	33.3	5.4	"

The cell concentration and morphology were noted after 4 days of growth. The standard concentration of red blood cell extract (3.3%) corresponds to 3.7 mg hemoglobin-protein per milliliter or 110–150 μg hemoglobin-heme per milliliter. The acriflavin concentration was 136 mμg/ml.

FLAVIN AND HEMIN:[4] Preliminary spectrophotometric evidence was obtained for the formation of a complex between acriflavin and hemin and also between acriflavin and protoporphyrin IX. The change in the absorption spectrum of acriflavin upon successive additions of equal amounts of hemin to both cuvettes is shown in Fig. 20. There is a decrease in absorbance at the maximum and a shift towards longer wavelengths; and there is also an appearance of new absorption bands at 575 and 635 mμ. The dye appears to be

[4] These experiments were performed in collaboration with Mr. Andrew Balber.

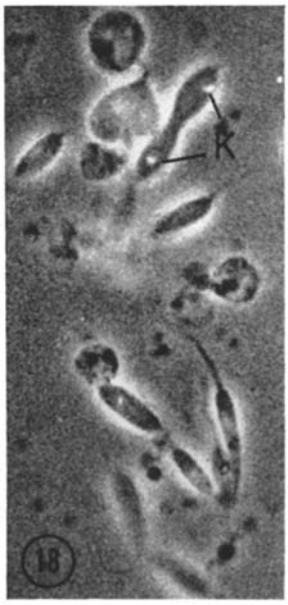

FIGURE 18 Kinetoplast (*K*) localization of acriflavin fluorescence. Cells treated with 47 mμg acriflavin per milliliter for 3 hr at 27°C in medium C with red blood cell extract and hemin (20 μg/ml). Simultaneous phase-contrast and fluorescence. × 800.

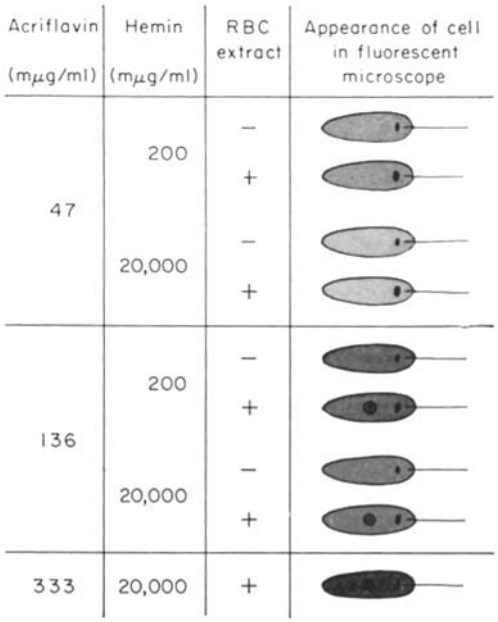

FIGURE 19 Schematic diagrams showing the effects of hemin and red blood cell extract on intracellular localization of acriflavin fluorescence. Cells were treated with acriflavin for 3 hr in medium C at 27°C and observed as air-dried, unfixed smears.

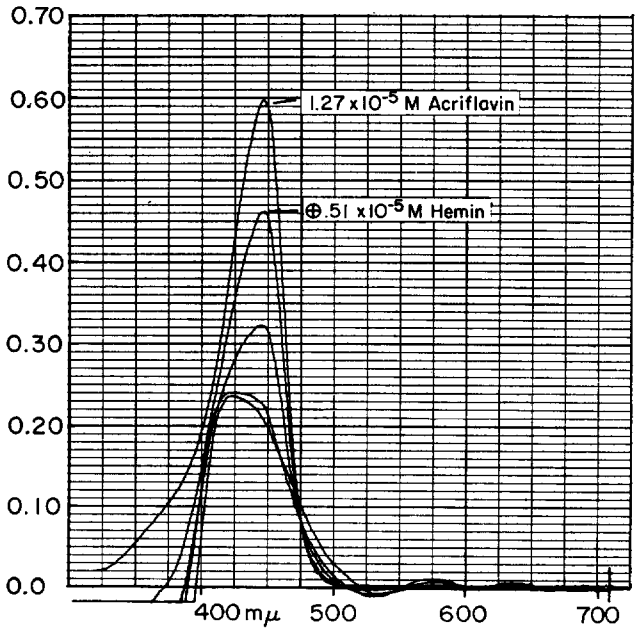

FIGURE 20 Effect of hemin on absorption spectrum of acriflavin (commercial). Successive equal amounts of hemin were added to both cuvettes. Purified acriflavin behaved identically.

saturated with hemin at a ratio of 1.2–1.6 moles of hemin per mole of acriflavin.

Similar absorbance changes can be seen in the spectra of hemin (Fig. 21) and protoporphyrin IX (Fig. 22) upon the successive addition of equal amounts of acriflavin to both cuvettes. The molecular nature of the complex is not known.

No change in the absorption spectrum of acri-

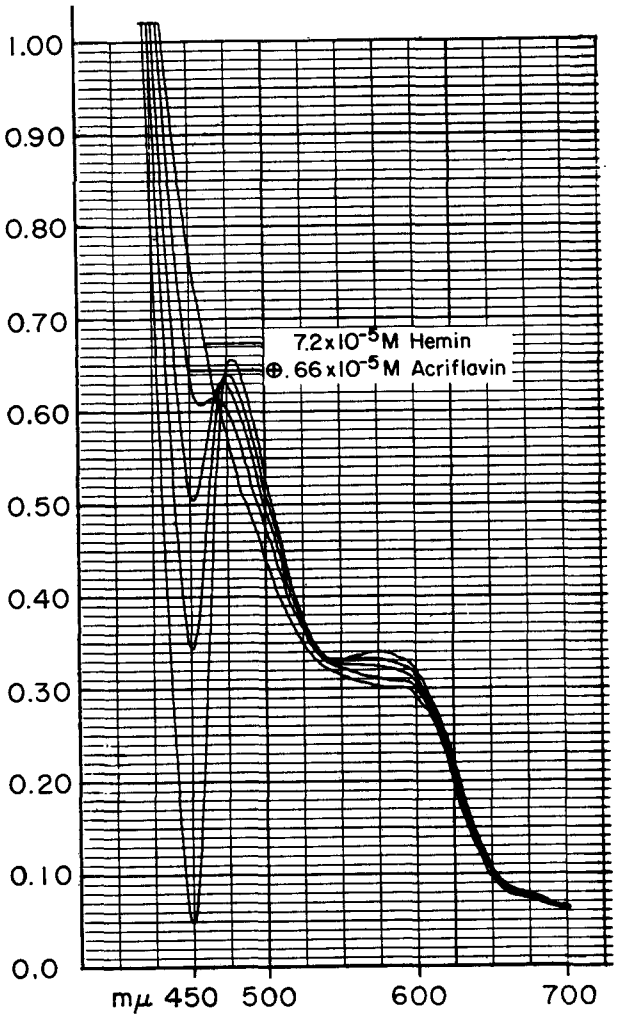

FIGURE 21 Effect of acriflavin on absorption spectrum of hemin. Successive equal amounts of acriflavin were added to both cuvettes.

flavin was noted upon the addition of red blood cell extract, hemoglobin, cytochrome C, or riboflavin to both cuvettes. This lack of complex formation between hemoglobin and acriflavin coincides with the ineffectiveness of hemoglobin in substituting for molecular hemin or protoporphyrin IX in the acriflavin effect. Probably the heme moieties in the hemoglobin molecule are sterically inaccessible to binding by acriflavin. The fact that riboflavin does not appear to bind to acriflavin makes it unlikely that the stimulation of production of dyskinetoplastic cells by high riboflavin concentrations in the presence of acriflavin, as noted by Trager and Rudzinska (1964), involves the same mechanism as that is involved in the case of hemin.

It therefore is probable that complex formation between hemin and acriflavin on the intracellular level is related to the observed loss of acriflavin fluorescence from the nucleus and cytoplasm. One possible mechanism may be a competition between hemin and acriflavin for the same intracellular binding sites. It is suggested as a working hypothesis that molecular hemin does not enter the kinetoplast and therefore has no effect on the dye bound to the kinetoplast DNA.

KINETICS OF UPTAKE OF ACRIFLAVIN:[4] The uptake of purified acriflavin was monitored by measuring the depletion of dye from the medium after removing the cells by centrifugation. Fairly high dye concentrations (3 μg/ml) were used in order to obtain readable optical densities.

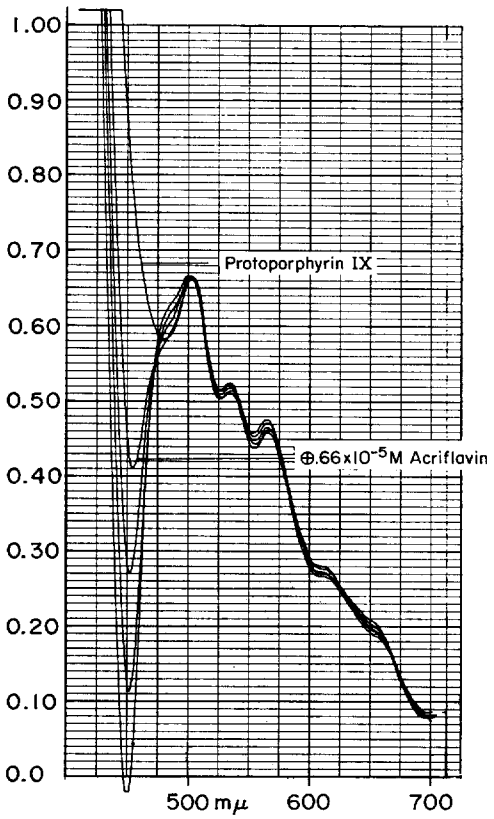

FIGURE 22 Effect of acriflavin on absorption spectrum of protoporphyrin IX. Same protocol as Fig. 21.

Cells became saturated with acriflavin in about 70 min at 27°C. That incubation at 0–4°C decreased the amount of dye taken up indicates that the uptake is an active process. This is demonstrated by Table V, which shows the amount of dye taken up at the saturation level by 10^8 cells under various conditions.[5]

Table V also shows that pretreatment of cells grown in low-hemin medium C (0.2 μg/ml) with high concentrations of hemin (10–30 μg/ml) decreases the total amount of acriflavin taken up on a per cell basis in hemin-free media. This may indicate that hemin binds to the same intracellular sites as acriflavin.

On the other hand, pretreatment of the cells with the standard concentration of red blood cell extract for 1 hr had no effect on the kinetics of uptake of acriflavin at 27°C. This agrees with the previous observation that red blood cell extract acts by increasing the total uptake of dye by the cells, presumably by inducing pinocytosis or a facilitated diffusion.

It should be noted that the acriflavin concentrations used for the uptake experiments were one to two orders of magnitude higher than those used to produce dyskinetoplastic cells, so that the data possibly may not be relevant. However, it is unlikely that the basic mechanism of dye uptake differs at different dye concentrations.

It is of some interest that high concentrations of acriflavin had a toxic effect only when cellular metabolism was active. Cells treated with 30 μg acriflavin per milliliter for 13 hr at 27°C were highly degenerate, whereas the same treatment at 4°C had no effect on morphology and motility.

TABLE V

Uptake of Acriflavin by L. tarentolae

Exp.	Hemin concentration during pretreatment	Moles purified acriflavin (× 10⁻⁹) taken up by 10^8 cells at the saturation level			
		27°C incubation		4°C incubation	
		Control	Hemin-treated	Control	Hemin-treated
	μg/ml				
1	10	6.6	—	0.58	—
2	20	16.2	—	7.7	—
3	30	17.3	6.2	—	—
4	10	5.0	4.2	2.9	1.2
5	10	5.0	4.2	2.5	2.2

Cells grown in low-hemin (0.2 μg/ml) medium C were preincubated for 1 hr in fresh high-hemin (10–30 μg/ml) medium C at 27°C. Control cells were similarly incubated in fresh medium C without hemin. Then both cultures were washed several times at 0–4° C in SBG and resuspended in SBG + 3 μg acriflavin per milliliter. 1 ml samples were immediately removed and centrifuged at 0–4°C for 10 min at 3000 rpm, to correct for the nonspecific, nonactive initial adsorption of dye. The control and experimental cultures were divided into two parts and incubated at 27° and 4°C. After the saturation level was attained (70 min), the cells were centrifuged down and the decrease in acriflavin concentration was measured (at $\lambda = 450$ mμ). The initial, nonspecific decrease was subtracted from the final decrease to obtain the above values. Five separate experiments are shown. The variation in the values of the controls at 27°C may reflect the varying physiological state of the cells in different experiments.

[5] Immediately upon mixing the cells with the dye at 0–4°C a nonspecific adsorption occurs, and a sample was removed to normalize the uptake curves.

Cells treated with 3 μg acriflavin per milliliter were unaffected after 3 hr at 27°C, so that the above uptake data were not due to toxicity phenomena.

LACK OF LOCALIZATION OF AN INACTIVE DYE, ACRIDINE ORANGE: Acridine orange was found to be inactive in the production of dyskinetoplastic *L. tarentolae*: it was merely inhibitory to cell growth. In agreement with this, the treatment of cells with low concentrations of acridine orange (< 300 mμg/ml) produced no preferential localization in the kinetoplast as seen in the fluorescence microscope. The dye was taken up equally well by the nucleus and kinetoplast, and was also visible in the cytoplasm.

Physiological Correlates of Loss of Kinetoplast DNA

LACK OF EFFECT ON CELL PROTEINS: No differences in the amounts of protein per cell could be detected by the Folin assay (Lowry et al., 1951). Stationary-phase normal and 70% dyskinetoplastic cells both gave a value of 2.4 μg protein per 10^6 cells.

Electrophoresis of total cell protein extracts from normal and 70% dyskinetoplastic cells gave identical patterns on cellulose polyacetate (Sepraphore III) (Fig. 23).

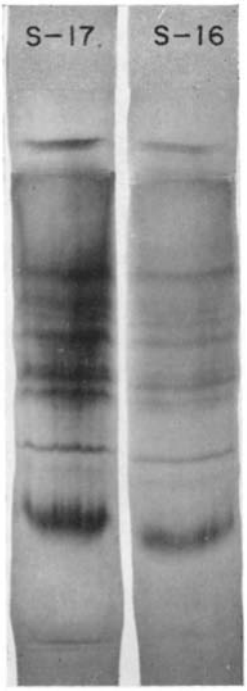

FIGURE 23 Electrophoretic comparison of proteins from total cell extracts of normal (S-16) and 70% dyskinetoplastic (S-17) *L. tarentolae*. Sepraphore III strips were stained with Ponceau S and destained in 5% acetic acid.

IMPAIRMENT OF PLATING EFFICIENCY ON BLOOD AGAR: *L. tarentolae* cells form colonies on blood agar. The colonies reach a maximum diameter of 1–3 mm by 10 days (Fig. 24). There is a great heterogeneity in colony size. The percentage of large colonies varied with the stage of the culture and the strain of cells used. Log-phase cells produced a greater percentage of large colonies and had a lower plating efficiency than stationary-phase cells. This may reflect a formation of large colonies from dividing forms or from several cells bound together in a rosette, a phenomenon which occurs with about a 10% frequency in log-phase cultures and which is rare in stationary-phase cultures. Cells from a large colony when replated, produced approximately the same percentage of large colonies as the stock cells.

The plating efficiencies of the untreated cells varied from 30 to 100%, depending on the age of the culture and the strain. As shown in Fig. 25, the plating efficiency of acriflavin-treated cells decreased greatly during growth of the culture. This decrease did not correlate linearly with the increase of dyskinetoplastic cells; it occurred more rapidly. All of the cells in randomly selected colonies from the dye-treated cells appeared normal with normal-sized kinetoplasts. It seems likely that the kinetoplast DNA is damaged after only a few divisions in the presence of the dye, and that those cells with damaged DNA cannot reproduce enough times to form colonies on agar. Such cells may indeed form microcolonies as in the case of "negative petites" in yeast (Bulder. 1964), but this was not investigated.

IMPAIRMENT OF RESPIRATION: The kinetoplast region of dyskinetoplastic cells does not take up the vital mitochondrial stain, Janus green B, as do normal kinetoplasts of untreated cells. In agreement with this observation, it was found that cellular respiration was progressively inhibited with growth in acriflavin. Fig. 26 compares the per cent inhibition of the QO_2 (μM O_2/min/10^7 cells) relative to an untreated control with the increase in percentage of dyskinetoplastic cells. The relationship is not linear, but this may be due to the crude method of

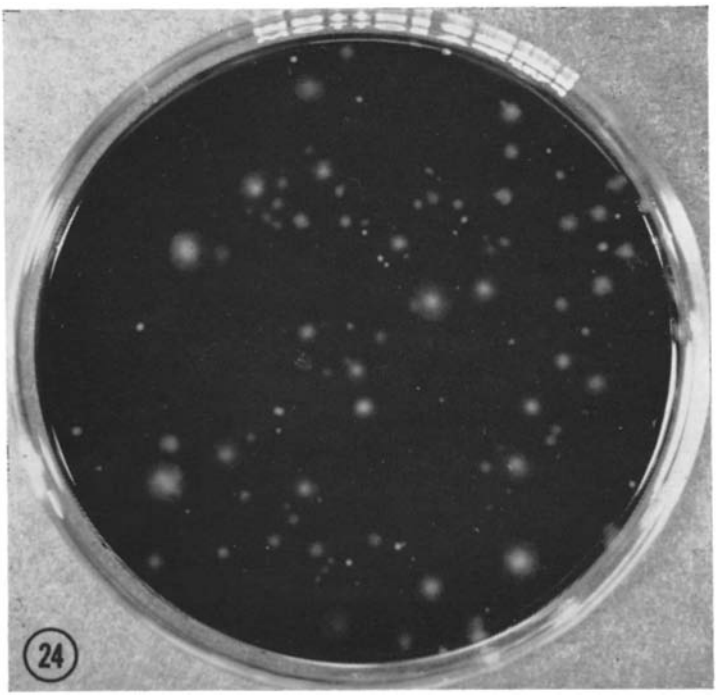

FIGURE 24. Colonies of *L. tarentolae* on blood agar. Plate is 9 inches in diameter. 10 days incubation at 27°C.

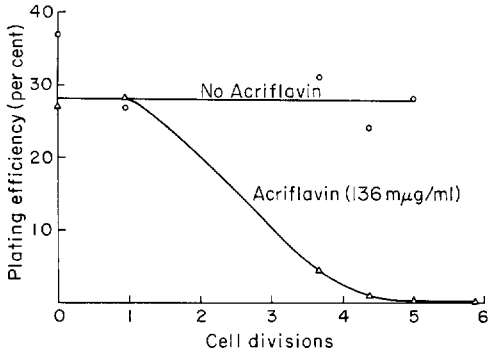

FIGURE 25 Decrease in per cent plating efficiency during growth in acriflavin (136 mμg/ml). Both control and experimental cells were in medium C with red blood cell extract and 20 μg hemin/ml.

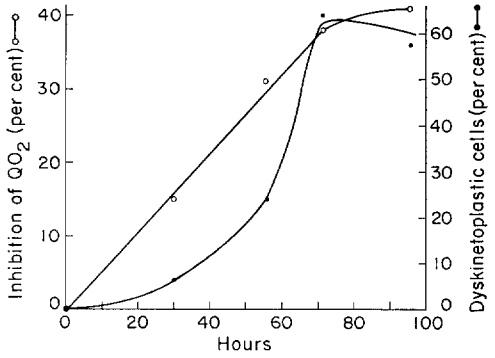

FIGURE 26 Inhibition of QO_2(μM O_2/min/10^7 cells) and increase in percentage of dyskinetoplastic cells during growth in acriflavin (136 mμg/ml). Cells were in medium C with red blood cell extract and 20 μg hemin/ml.

scoring a cell as dyskinetoplastic when no stainable kinetoplast DNA is left. The respiration of the dye-treated 70-hr cells was sensitive to KCN, antimycin A, and Na amytal at approximately the same levels as the untreated cells. However, the oxygen uptake of dye-treated 70-hr cells was nonlinear with respect to oxygen concentration even at 80–90% oxygen levels. This nonlinearity was not due to cell death because the slope returned to the original value upon bubbling air

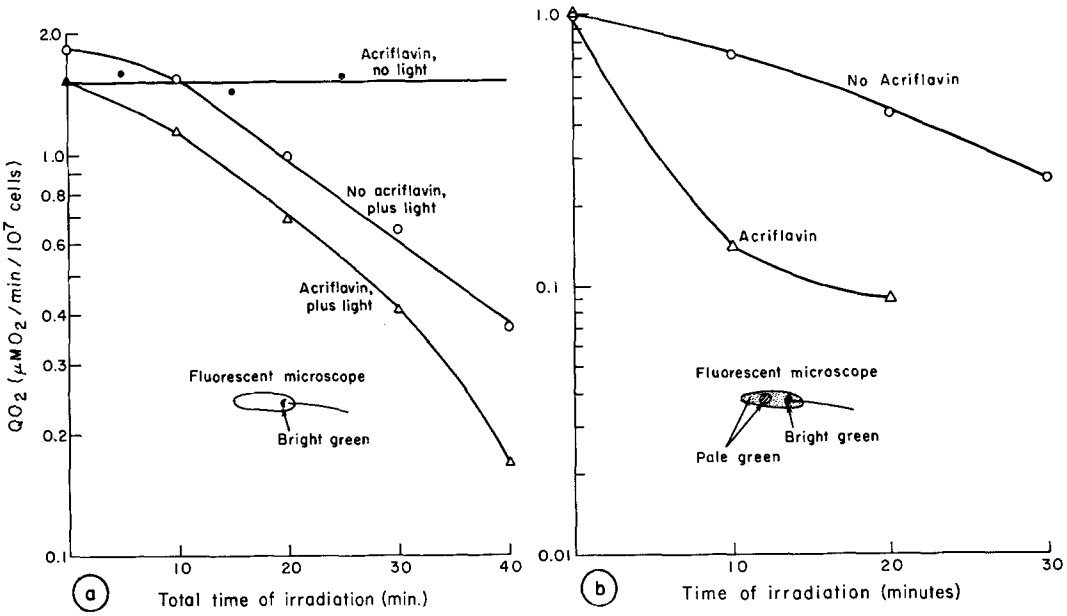

FIGURE 27 Effect of visible irradiation of acriflavin-treated and untreated cells. Cells were pretreated with high (*a*) and low (*b*) acriflavin concentrations and then washed. Cells were irradiated in acriflavin-free SBG for 5-min periods at 20°C with stirring, and then the QO_2 was measured with an oxygen electrode. The abscissa represents total irradiation time, the inhibition being irreversible and additive. The no light, plus acriflavin control in *b* was identical with that in *a* and was omitted from the graph.

through the liquid. It possibly indicates a damage to cytochrome oxidase in its ability to bind molecular oxygen.

Dyskinetoplastic cells could not be subcultured in rich media such as blood agar, even with the addition of high levels of glucose. Usually, normal cells would grow out after a variable lag period.

Possible Use of Selective Localization of Acriflavin in the Kinetoplast to Produce Kinetoplast Mutations

SELECTIVE PHOTOOXIDATION OF KINETOPLAST DNA: Since acriflavin binds fairly selectively to the kinetoplast DNA under the proper cultural conditions, and since acriflavin is a good photosensitizer, it may be useful to selectively photooxidize the kinetoplast DNA. One could then select for kinetoplast mutants among the survivors by agar-plating techniques. In a preliminary study, the effects of visible irradiation of acriflavin-treated *L. tarentolae* were investigated in terms of respiration and plating efficiency. Fig. 27 shows that such irradiation produces an immediate irreversible damage to cellular respiration, an effect that is accentuated at higher dye concentrations. Visible irradiation in the absence of the dye also damages respiration, but there is a definite accentuation of the effect by prior treatment with acriflavin. The decrease in respiration correlated well with decrease in cell motility.

The plating efficiency of dye-treated, irradiated cells also decreased. 10 min of visible irradiation of cells which had the dye apparently bound exclusively in the kinetoplast decreased the plating efficiency on blood agar from 29 to 0.7%.

This photodynamic action was apparent only with high light intensities; ordinary room illumination had no effect on respiration or on the production and viability of dyskinetoplastic cells by growth in the presence of acriflavin.

DISCUSSION

The initial effect of acriflavin on *L. tarentolae* is a rapid selective inhibition of kinetoplast DNA synthesis, with very little effect on nuclear DNA synthesis or on cell and kinetoplast division. This results in a decrease in the average amount of DNA per kinetoplast for two to three generations, and then a cell division occurs in which one daughter cell retains all the kinetoplast DNA and

one dyskinetoplastic cell is produced. The fact that the dyskinetoplastic cells continue to divide, although at a lower rate than normal cells, has interesting implications for the control of the formation of mitochondrial structure, if it can be firmly established that there is no mitochondrial DNA left in dyskinetoplastic cells.

Several correlations were established between the selective effect of acriflavin on kinetoplast DNA synthesis and the intracellular localization of acriflavin as seen by fluorescence microscopy. It is recognized that the visual brightness of acriflavin fluorescence need not be related directly to the amount of dye present, owing to possible perturbations in the fluorescence caused by the local environment of the dye molecules. However, the correlations obtained seem to imply a causal relationship between the apparent intracellular localization of dye and the effect on cell growth and organelle development. For example, whenever it was observed that the dye localized fairly exclusively in the kinetoplast, a high percentage of dyskinetoplastic cells was obtained after several cell divisions; if the dye was seen in the nucleus and cytoplasm as well as in the kinetoplast, cell division was inhibited, and a lower percentage of dyskinetoplastic cells was obtained.

The localization of acriflavin in the kinetoplast was a function of dye concentration and the composition of the culture medium. Low dye concentrations favored a kinetoplast localization, and addition of a high concentration of hemin stimulated this phenomenon. There are several possible explanations for the selective binding of acriflavin to the kinetoplast, even in hemin-free media. The higher adenine-plus-thymine base composition of the kinetoplast DNA should favor binding of acriflavin (Tubbs et al., 1964; Guttman and Eisenman, 1965), but probably this can not account for the large differences observed. The absence of histones in the kinetoplast (Steinert, 1965) and their presence in the nucleus may be another factor favoring kinetoplast binding. The failure to recover the expected amount of kinetoplast DNA in phenol extractions may also be relevant to this question. Finally, differences in ionic concentrations in the two organelles may influence the binding of acriflavin. This must remain an open question.

The mechanism by which high concentrations of hemin, a necessary constituent of trypanosome culture media, produce a kinetoplast localization of acriflavin is unknown, but it does seem, somehow, to be related to the ability of hemin to form a complex with acriflavin, for two reasons: (*a*) protoporphyrin IX, which cannot be used nutritionally by *L. tarentolae*, both forms a complex with acriflavin and produces a kinetoplast localization of dye; (*b*) hemoglobin, conversely, does not form a complex with acriflavin and does not produce a kinetoplast localization of dye.

Another correlation demonstrated involved the increase in intensity of fluorescence of intracellular dye when hemoglobin was present in the medium. This was interpreted as an increased uptake of the dye caused by a stimulation of pinocytosis or facilitated diffusion by the hemoglobin protein, and could be correlated with an increased sensitivity to the dye as regards the inhibition of cell division and the production of dyskinetoplastic cells. The increased production of dyskinetoplastic cells may be due in part to another mechanism, a stimulation of the further division of already dyskinetoplastic cells by the added nutrients, but this was not investigated.

The mechanism of selective inhibition of kinetoplast DNA synthesis and its subsequent dilution by cell division is probably valid for other species of hemoflagellates also. However, to explain the reported initial all and none kinetoplast division that occurs in *Crithidia fasciculata* (Cosgrove, 1966), *Trypanosoma lewisi*, *T. equiperdum*, and *T. brucei* (Jirovec, 1929), one must assume that the dye interferes with the mechanism for distributing the kinetoplast DNA between daughter kinetoplasts as well as with kinetoplast DNA replication. This indeed may be a function of the 100-fold higher levels of acriflavin necessary to produce dyskinetoplastic cells in these species.

Several physiological correlates of the loss of kinetoplast DNA in *L. tarentolae* were demonstrated. It should be stressed, however, that owing to the lethal nature of the dyskinetoplastic state in this species, it is difficult to dissociate general pathological symptoms from events more closely related to the loss of kinetoplast DNA. The correlates obtained, an inhibition of respiration and a decrease in plating efficiency, agree with the hypothesis that the loss of kinetoplast DNA produces a respiratory defect which leads to a decrease in biosynthetic reactions and eventual cell death.

The rapid decrease in plating efficiency prior to the complete loss of kinetoplast DNA agrees with the electron microscopic evidence of Trager and Rudzinska (1964) that the first visible effect of acriflavin on the kinetoplast DNA is a clumping of

its fibrillar structure. The damage, however, does not seem to render the kinetoplast DNA incapable of initially being normally distributed among progeny kinetoplasts.

The apparent lack of gene recombination in the hemoflagellates eliminates classical genetic analysis as a tool to study the kinetoplast. It still might be possible, however, to obtain viable kinetoplast mutants and study their properties. The ability of hemoflagellates to form colonies on agar (Nöller, 1917; Packchanian and Kelley, 1967) would permit the selection of mutant clones by using markers such as temperature sensitivity, drug resistance. or nutritional abilities. A possible method for the production of kinetoplast mutants in *L. tarentolae* was proposed, which involves the selective photooxidation (Friefelder et al., 1961; Simon and Van Vunakis, 1963) of the kinetoplast DNA by visible light. The phenomenon of dye-induced sensitization of hemoflagellates to visible light has been known since the work of Busck and Tappeiner (1906). The first observations by fluorescence microscopy that dyes such as acriflavin apparently concentrated in the kinetoplast of trypanosomes were made by Von Jansco (1931, 1932), who showed that the uptake of dye into the kinetoplast correlated well with the advent of photosensitivity. Walker (1961) showed that selective irradiation of the kinetoplast of an acriflavin-treated trypanosome with visible light produced cell death, whereas similar irradiation of the nucleus had no effect. It seems likely that the preferential localization of the dye in the kinetoplast was causally related to the cell damage produced by visible irradiation. In the attempts to obtain kinetoplast mutants by the method of photodynamic action, it could always be argued that damage to the nuclear DNA mediated by dye molecules bound to the nucleus produced the mutations. Walker's (1961) experiment makes this seem unlikely, but does not completely eliminate the possibility.

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REFERENCES

ALBERT, A. 1966. The Acridines. St. Martin's Press, Inc., New York. 2nd edition.

ANDERSON, W. A., and R. A. ELLIS. 1965. Ultrastructure of *Trypanosoma lewisi*: Flagellum, microtubules and the kinetoplast. *J. Protozool.* **12**:483.

BRESSLAU, E., and L. SCREMIN. 1924. Die Kerne der Trypanosomen und ihr Verhatten zur Nukleal-reaktion. *Arch. Protistenk.* **48**:509.

BULDER, C. J. E. A. 1964. Lethality of the petite mutation in petite negative yeasts. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **30**:442.

BURTON, K. 1956. A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric determination of DNA. *Biochem. J.* **62**:315.

BUSCK, G., and H. v. TAPPEINER. 1906. Über Lichtbehandlung blutparasitarer Krankheiten. *Deutsch. Arch. Klin. Med.* **87**:98.

CARO, L. 1964. High resolution autoradiography. *In* Methods in Cell Physiology. D. Prescott, editor. Academic Press Inc., New York. **2**.

CLARK, T. B., and F. B. WALLACE. 1960. A comparative study of kinetoplast ultrastructure in the Trypanosomatids. *J. Protozool.* **7**:115.

COSGROVE, W. B. 1966. Acriflavin-induced akinetoplasty in *Crithidia fasciculata. Acta Protozool.* **4**:155.

DUBUY, H. G., C. F. T. MATTERN, and F. L. RILEY. 1965. Isolation and characterization of DNA from kinetoplasts of *Leishmania enrietti. Science.* **147**:754.

DUBUY, H. G., C. F. T. MATTERN, and F. L. RILEY. 1966. Comparison of the DNAs obtained from brain nuclei and mitochondria of mice and from the nuclei and kinetoplasts of *Leishmania enrietti. Biochim. Biophys. Acta.* **123**:298.

EVANS, T. E. 1966. Synthesis of a cytoplasmic DNA during the G_2 interphase of *Physarum polycephalum. Biochem. Biophys. Res. Commun.* **22**:678.

FREIFELDER, D., P. F. DAVISON, and E. P. GEIDUSCHEK. 1961. Damage by visible light to the acridine orange-DNA complex. *Biophys. J.* **1**:389.

GUDE, W. D., A. C. UPTON, and T. T. ODELL, Jr. 1955. Giemsa staining of autoradiographs prepared with stripping film. *Stain Technol.* **30**:161.

GUTTMAN, H. N., and R. N. EISENMAN. 1965. Acriflavin-induced loss of kinetoplast DNA in *Crithidia fasciculata* (*Culex pipiens* strain). *Nature.* **207**:1280.

HONIGSBERG, B. M., W. BALAMUTH, E. C. BOVEE, J. O. CORLISS, M. GOJDICS, R. P. HALL, R. R. KUDO, N. D. LEVINE, A. R. LOEBLICH, J. WEISER, and D. H. WEINRICH. 1964. A revised classification of the Phylum Protozoa. *J. Protozool.* **11**:7.

INOKI, S., K. NAKANISHI, and T. NAKABAYASHI. 1958. Study of *Leishmania donovani* with special reference to the kinetoplast, mitochondrial, and

golgi zone by electron microscopy employing the thin section technique. *Biken's J.* **1**:194.

JIROVEC, O. 1929. Studien über blepharoplastlose Trypanosomen. *Arch. Protistenk.* **68**:187.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARE, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265.

MESELSON, M., F. W. STAHL, and J. VINOGRAD. 1957. Cesium chloride gradient centrifugation. *Proc. Natl. Acad. Sci. U.S.* **43**:581.

MÜHLPFORDT, H. 1963. Über die Bedeutung und Feinstruktur des Blepharoplasten bei parasitischen Flagellaten. **1**:14; **2**:357, 476.

NEUBERT, D., H. HELGE, and R. BASS. 1965. Einbau von thymidin in die DNA von mitochondrien. *Arch. Exptl. Pathol. Pharmakol.* **252**:258.

NEUFELD, H. A., A. N. LEVAY, F. V. LUCAS, A. P. MARTIN, and E. STOLZ. 1958. Peroxidase and cytochrome oxidase in rat tissues. *J. Biol. Chem.* **233**:209.

NOLLER, W. 1917. Blut-und Insektenflagellatenzuchtung auf Platten. *Arch. Schiffs Tropenhyg.* **21**:53.

ORMEROD, W. E. 1961. The study of volutin granules in trypanosomes. *Trans. Roy. Soc. Trop. Med. Hyg.* **55**:313.

PACKCHANIAN, A., and L. KELLEY. 1967. Colonial growth and morphology of trypanosomes, leishmanias and leptomonads. I. Studies on *Endotrypanum schaudinni*. *Texas Rep. Biol. Med.* **24**:639.

PARSONS, J. A. 1965. Mitochondrial incporporation of H^3-thymidine in *Tetrahymena pyriformis*. *J. Cell Biol.* **25**:641.

RABINOWITSCH, L., and W. KEMPNER. 1899. Beitrag zur Kenntnis der Blutparasiten, speziell der Rattentrypanosomen. *Z. Hyg.* **30**:251.

RIOU, G., R. PAUTRIZEL, and A. PAOLETTI. 1966. Fractionnement et caractérisation de l'ADN de Trypanosome (*T. equiperdum*). *Compt. Rend.* **262**: D 2376.

RIS, H. 1962. Ultrastructure of certain self-dependent cytoplasmic organelles. Electron Microscopy: Fifth International Congress on Electron Microscopy Held in Philadelphia, Pennsylvania, August 29 to September 5, 1962. S. S. Breese, Jr., editor. Academic Press Inc., New York. **2**:XX-1.

RUDZINSKA, M. A., P. A. D'ALESANDRO, and W. TRAGER. 1964. The fine structure of *Leishmania donovani* and the role of the kinetoplast in the leishmania-leptomonad transformation. *J. Protozool.* **11**:166.

SAITO, H., and K. MIURA. 1963. Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochim. Biophys. Acta.* **72**:619.

SCHILDKRAUT, C., J. MARMUR, and P. DOTY. 1962. Determination of the base composition of DNA from its buoyant density in cesium chloride. *J Mol. Biol.* **4**:430.

SCHULZ, H., and E. MACCLURE. 1961. Electron microscopische Untersuchung des *Trypanosoma cruzi* mit besonderer Berücksichtigung des Periplasten und Blepharoplasten. *Z. Zellforsch. Mikroskop. Anat.* **88**:389.

SHACK, J., and W. M. CLARK. 1947. Metalloporphyrins. VI. Cycles of changes in systems containing heme. *J. Biol. Chem.* **171**:143.

STEINERT, M. 1960. Mitochondria associated with the kinetonucleus of *Trypanosoma mega*. *J. Biophys. Biochem. Cytol.* **8**:542.

SIMON, M. I., and H. VAN VUNAKIS. 1963. The photodynamic reaction of methylene blue with deoxyribonucleic acid. *J. Mol. Biol.* **4**:488.

STEINERT, M. 1964. Le Chondriome de *Trypanosoma mega*, *J. Cell Biol.* **20**:192.

STEINERT, M. 1965. L'Absence d'histone dans le kinétonucléus des Trypanosomes. *Exptl. Cell. Res.* **39**:69.

STEINERT, M., and G. STEINERT, 1962. La synthèse de l'acide désoxyribonucléique au cours du cycle de division de *Trypanosoma mega*. *J. Protozool.* **9**:203.

TRAGER, W. 1957. Nutrition of a hemoflagellate (*Leishmania tarentolae*) having an interchangeable requirement for choline or pyridoxal. *J. Protozool.* **4**:269.

TRAGER, W., and M. A. RUDZINSKA. 1964. The riboflavin requirement and the effects of acriflavin on the fine structure of the kinetoplast of *Leishmania tarentolae*. *J. Protozool.* **11**:133.

TUBBS, R. K., W. E. DITMARS, JR., and Q. VAN WINKLE. 1964. Heterogeneity of the interaction of DNA with acriflavin. *J. Mol. Biol.* **9**:545.

VICKERMAN, K. 1962. The mechanism of cyclical development in trypanosomes of the *T. brucei* subgroup. *Trans. Roy. Soc. Trop. Med. Hyg.* **56**:487.

VON JANSCO, N. 1931. Photobiologische Studien in der Chemotherapie I & II. *Zentr. Bakteriol. Parasitenk. Abt. II. Orig.* **122**:388, 393.

VON JANSCO, N. 1932. Beobachtung chemotherapeutischer Vorgänge im Fluorescenzmikroskop. *Klin. Wochschr.* **11**:689.

WALKER, P. J. 1961. Organization of function in trypanosome flagellum. *Nature.* **189**:1017.

WERBITZKI, F. W. 1910. Über blepharoplastlose Trypanosomen. *Zentr. Bakteriol. Parasitenk. Abt I. Orig.* **53**:303.