ACRIFLAVIN RESISTANCE IN THE HEMOFLAGELLATE, *LEISHMANIA TARENTOLAE*

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ABSTRACT

The accumulation, metabolism, and distribution of acriflavin (acr) in two culture strains of *Leishmania tarentolae* were studied. One strain, reported previously, was sensitive to the dye, i.e. became dyskinetoplastic and could not be subcultured in the presence of 470 ng/ml acr, and one was resistant. Accumulation was studied by fluorescence of the dye within cells and by uptake of acr-^{3}H by cells. Metabolism was studied by paper chromatography of aqueous extracts from cells grown with acr-^{3}H, and distribution was examined by fluorescence and quantitative electron microscope radioautography. Substances affecting the response to acr included hemin and an acr-sensitizing factor initially obtained from red cells but here shown to be distinct from hemoglobin. In the presence of the sensitizing factor or in the absence of hemin, the resistant strain became dyskinetoplastic and could not be subcultured. Acr fluorescence appeared in the nucleus of the resistant strain, and the percentage of radioautography grains appearing in the nucleus increased. Under these conditions the distribution of radioactivity from chromatographed extracts was altered from the normal in a similar fashion. Because sensitization of the resistant strain is associated with increased amounts of acr in the nucleus, that organelle may be implicated in the mode of action of acr. In general, the two strains behaved alike except for (*a*) the response to acr, (*b*) the arginine requirement for optimal growth, and (*c*) the sensitivity to cycloheximide. Thus, one cannot exclude the wider possibility that acr may act on the cytoplasm and the nucleus as well as on the mitochondrion.

INTRODUCTION

Acriflavin (2,3 - diamino - *N* - methylacridinium chloride) inhibits mitochondrial as opposed to nuclear DNA synthesis (Meyer and Simpson, 1969; Fukuhara and Kujawa, 1971; Lizardi, 1971). In hemoflagellates it causes the kinetoplast[1] to disappear (Mühlpfordt, 1964; Trager and Rudzinska, 1964; Cosgrove, 1966; Steinert and Van Assel, 1967; Simpson, 1968; Hill and Anderson, 1969; Strauss, 1971 *b*). The loss of the kinetoplast is usually termed dyskinetoplasia (Trager and Rudzinska, 1964; see Figs. 1 and 2). The compact coil of mitochondrial DNA seen in thin sections of the kinetoplast nucleoid becomes disarrayed and finally remains only as a "smudge." (See Figs. 8–12). There are also indications that acriflavin (acr) may interact at other loci in the cell's metabolism (Strauss, 1971 *b*). An acriflavin-resistant strain of *Leishmania tarentolae* has been described in which resistance is removed by a substance from red cells. In the presence of the substance, fluorescence of the

[1] The kinetoplast of hemoflagellates is that area of the mitochondrion containing the mitochondrial DNA (K-DNA). K-DNA comprises about 20% of the cell's DNA and can be seen in Giemsa- or Feulgen-stained preparations as a dense body at the base of the flagellum.

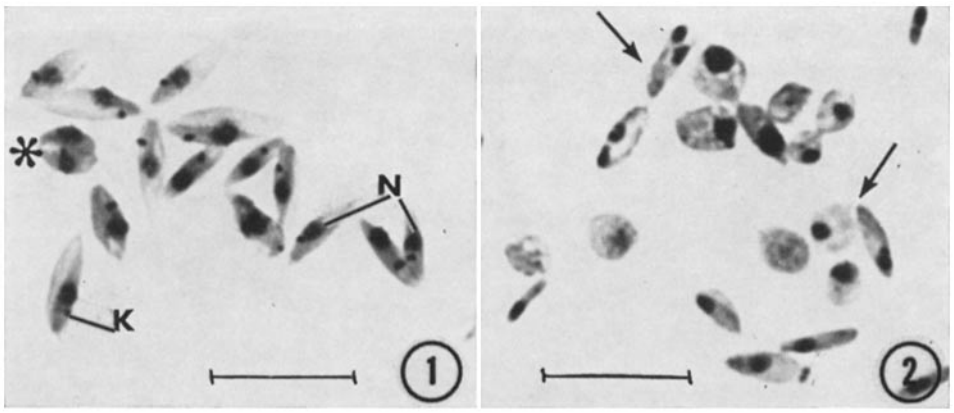


FIGURE 1 *L. tarentolae* resistant to 470 ng/ml purified acr continuously subcultured for more than 12 months in the presence of dye. Stain: Giemsa. *K*, kinetoplast; *N*, nucleus; ⁎, dividing cell. Light micrograph. Bar indicates 10 μ. × 1900.

FIGURE 2 *L. tarentolae* sensitive to 470 ng/ml acr 5 days after inoculation into medium containing acr. Stain: Giemsa. Arrows indicate dyskinetoplastic cells. Light micrograph. Bar indicates 10 μ. × 1900.

dye, which normally appears only in the kinetoplast region, appears in the nucleus and cytoplasm as well.

Although the proposed mechanism of action of acr involves intercalation with DNA as demonstrated in vitro (Lerman, 1961, 1963; Tubbs et al., 1964), the only direct evidence for intercalation occurring in vivo is the fluorescence localization in hemoflagellates mentioned above (Simpson, 1968; Strauss, 1971 *b*). Fluorescence, however, is an unreliable indicator of where a substance is located, as the local microenvironment of the bound dye can drastically affect its fluorescent properties and, hence, its apparent localization and amount (Udenfriend, 1962, 1969; Silver, 1967; Silver et al., 1968). Moreover, it would be remarkable if acr, which can bind to protein (Glazer, 1965; Bernhard et al., 1966), polysaccharide (Saunders, 1964; Braun, 1965), transfer RNA (Werenne et al., 1966; Weinstein and Finklestein, 1967), and nucleotides (Nicholson and Peacocke, 1966) as well as to DNA, should interact in the cell exclusively with mitochondrial DNA.

The following study extends the comparison of the acr-sensitive and acr-resistant strains of *L. tarentolae*. It describes the outcome of quantitative electron microscope radioautography employing acr-^{3}H to better assess the localization of the dye in the cell. It also provides further information on the acr-sensitizing factor.

METHODS

Culture

Promastigotes[2] of *L. tarentolae* were grown in medium C, (Trager, 1957), as 3.0 ml stationary cultures. Unless otherwise specified, hemin was present at a final concentration of 20 μg/ml. Hemin was solubilized by bringing a 0.2% solution in 0.05 N NaOH to a boil and filtering it through a Millipore filter (Millipore Corporation, Bedford, Mass.) with 0.45 μ pore size. Stock cultures were transferred every 7 days. Other experiments were ended or transferred between 4 and 7 days. Since the strain resistant to acriflavin (A strain) required 5 days to reach stationary phase, experiments involving it were ended or transferred between 5 and 7 days, unless otherwise noted. Methods for counting and for determining dyskinetoplasia have been described previously (Simpson, 1968). Only those cells with no stainable kinetoplast whatsoever were counted as dyskinetoplastic.

The resistant strain was obtained by continuously subculturing the parasite in progressively higher concentrations of acriflavin (acr) (Strauss, 1971 *b*). Resistance, which became manifest 4–6 months after the onset of exposure to the dye, was operationally defined as the differential ability to be subcultured continuously in the presence of 470 ng/ml acr. Resist-

[2] The promastigote form, found in the insect vector in nature, is the only form of *L. tarentolae* which has been cultured successfully in a defined medium (see Fig. 1).

ance was maintained for more than 4 months in the absence of the dye. Its loss could be explained as reversion to sensitivity of one or a few parasites and subsequent overgrowth by the revertant. It will be shown in Results that the sensitive strain (T) entered log phase somewhat sooner than the A strain and often grew to slightly higher cell concentrations than did A.

Acriflavin Purification and Preparation of Tritiated Acriflavin

Throughout the study acr freed of proflavin was used. Purification was achieved, as previously reported by Simpson (1968), by the method of Albert (1966). Purity of the product was determined by comparing its visible spectrum and its behavior upon paper chromatography in two different solvent systems, butanol:acetic acid (7:3) and 3% aqueous NH_4Cl, with a standard previously prepared in this laboratory. Neither pure proflavin nor the standard of purified acr moved in the butanol/acetic acid system with the rate of flow (R_f) described by Albert. Instead, the R_f for acr in our hands was 0.63 and the R_f for proflavin was 0.67. The R_f's for acr and proflavin in NH_4Cl were the same as those reported by Albert.

25 mg purified acr was tritiated by New England Nuclear Corp. (Boston, Mass.) by means of catalytic exchange over palladium in glacial acetic acid. The resulting material, 19 mCi/mg, free of unbound tritium, was freed from other impurities by ascending paper chromatography in 3% NH_4Cl. The brightly fluorescing band just above the origin was eluted from the paper with methanol and dried by flash evaporation. It was then resuspended in water and rerun against standards in both chromatography systems. In each case a single peak of radioactivity was observed when the strips were scanned with a Packard Radiochromatogram Scanner (Packard Instrument Co., Inc., Downers Grove, Ill.). It corresponded to the colored spot left by the standard. There was no evidence of proflavin or hydrogenated acridinium compounds by the visible spectrum or by movement on either chromatography system. The specific activity of the repurified material, based on its absorbance at 450 nm, was 780 mCi/mmole.

Isotope Extraction from Cells

To determine whether the isotope incorporated by the cells grown under various conditions was still acr, cells were grown for 5 days in the presence of 500 ng/ml acr, final specific activity 600 μCi/μg, washed 5 times with saline, and lysed by the addition of 2% aqueous sarkosyl (Geigy Chemical Corp., Ardsley, N. Y.). A portion of washed cells was also dissolved in Protosol (New England Nuclear Corp.). Portions of the supernatant from the sarkosyl lysate and of the Protosol solutions were counted in a Packard liquid scintillation counter (3003) with Bray's scintillation fluid (Bray, 1960). In this manner it was shown that all the cell-bound counts could be recovered. The sarkosyl lysate was run on ascending paper chromatography in both NH_4Cl and butanol/acetic acid systems and compared with acr-^{3}H in sarkosyl run at the same time.

Microscopy and Electron Microscope Radioautography

The method for preparing cells for electron microscopy has been described previously (Strauss, 1971 *a*). Specimens were examined with an RCA-3F electron microscope at 50 kv. For EM radioautography, cells were grown in 500 ng/ml acr-^{3}H, final specific activity 600 μCi/μg, for 2 or 5 days. They were fixed as described above by adding buffered glutaraldehyde directly to the cellular suspension. In order to determine that glutaraldehyde did not artifactually affix acr to the exterior surfaces of cells, a wash-out experiment was done. Cells were fixed with buffered glutaraldehyde directly in defined medium containing isotope and washed 4 times with saline-buffered glucose (SBG) (Simpson, 1968) or they were fixed after 4 washes with SBG. Portions from the fixation supernatant and from each wash were counted. The wash-out curves were identical. During one fixation, portions were removed at each step and counted. 15% of the isotope initially found with cells had been extracted, half the loss occurring at the final step in processing, i.e., while the cells remained in propylene oxide–Epon overnight in the cold. Pale gold sections were treated according to Salpeter (Salpeter and Bachmann, 1964; Salpeter, 1966), using Ilford L-4 nuclear track emulsion (Ilford Ltd., Ilford, England). For some experiments, before being dipped, the sections were stained and then carbon coated to prevent dense deposits, destaining, and loss of sensitivity of the emulsion in contact with the specimen (Salpeter and Bachmann, 1964). Even when the stained sections were carbon coated, however, there was considerable deposit over large areas. To avoid the deposits in later experiments, the sections were not stained, obviating the necessity for the carbon coat.

Slides were developed periodically up to 3.5 months after dipping as described by Salpeter (1966). 3–3.5 months provided adequate exposure for all experiments.

Grains were photographed at × 5500, every grain in the entire grid square being photographed. Bias due to observing grains in only one cell was not a problem because each section contained cross-sections of many cells. Background counts were less than 6 grains/grid square, while grains/grid square over

sections always exceeded 18 and were often as high as 72. The plates were examined on a light box and grain counts were made with a Bausch and Lomb measuring magnifier (Bausch and Lomb Inc., Scientific Instrument Div., Rochester, N. Y.) with a metric scale. The metric scale was used for observing the probability diameters around each grain, described by Bachmann et al. (1968) and Salpeter et al. (1969). Grain centers were arbitrarily chosen as close to the actual center as possible. At least 1200 grains were counted for each experimental determination. Relative volumes of organelles were determined by the method of Weibel et al. (1966). Thin sections were prepared from the sets of blocks used for radioautography. 50 random areas from each experiment were photographed and projected to a known magnification onto grid pattern C (Weibel et al., 1966).

Acriflavin-Sensitizing Factor

Red cell extract (RCE) was prepared as described by Simpson (1968) by freeze-thawing, except that it was not centrifuged to remove cellular debris. It was stored sterile at −20°C. Commercial hemoglobin was obtained from two sources. Equine hemoglobin, twice recrystallized, was obtained from Pentex Biochemical (Kankakee, Ill.). Bovine hemoglobin, twice recrystallized and dialyzed, was obtained from Sigma Chemical Co. (St. Louis, Mo.).

TABLE I

Comparison of Sensitive (T) and Resistant (A) Strains of L. tarentolae

Condition	Presence of: Acriflavin (470 ng/ml)	Presence of: RCE	Response of: A	Response of: T
Growth properties				
Lag time	+ or −	+ or −	36 hr	24 hr
Population doubling time in log phase	−	−	12 hr	12 hr
	+	−	12 hr	12 hr; 24 hr*
	+	+	21.6 hr	21.6 hr
Dyskinetoplasia after 5–7 days growth	−	−	<10%	<10%
	+	−	<10%	≥40%
	+	+	>10%	≥40%
Hemin requirement for ≥50% growth	−	−	<613 nM	<613 nM
	+	−	31 mM	≥ 31 mM
	+	+	31 mM	> 31 mM
Arginine requirement	−	−	184 μM—continuous growth	46 μM—continuous growth
	+	−	184 μM—continuous growth	not done
Chloramphenicol 2.2 mM	−	−	excellent growth	fair growth
	+	−	death at subculture (no D)	not done
1.1 mM	−	−	excellent growth	excellent growth
	+	−	excellent growth	death at subculture and D
Cycloheximide 1 nM	−	−	death at subculture (no D)	continuous growth
	+	−	death at subculture	death at subculture
0.25 nM	−	−	continuous growth	same as A
Ethidium bromide 272 nM	−	−	continuous growth	death and D

* Initial population doubling time: 12 hr. Population doubling time between 36 and 72 hr: 24 hr.

Column chromatography with the use of Sephadex G100 (Pharmacia Fine Chemicals Inc., Uppsala, Sweden) in a 90 × 2 cm column was performed on Hb-Pentex at 4°C. The effluent was monitored at 280 and 406 nm and occasionally at 540 nm on a Beckman DU monochrometer (Beckman Instruments, Inc., Fullerton, Calif.) with Gilford automatic recording spectrophotometer attachments (Gilford Instrument Laboratories, Oberlin, Ohio). Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin (Mann Research Labs, Inc., New York) as standard. Fractions were assayed for biological activity by adding portions to the resistant strain in the presence of 470 ng/ml acr. After 5 days, cells were counted and dyskinetoplasia was determined as described above.

Hb-Pentex was also chromatographed at room temperature on diethylaminoethyl (DEAE)-Sephadex A50 in 0.05 M Tris (Huisman and Dozy, 1965; Huisman et al., 1967; Dozy et al., 1968). The sample was dissolved in Tris 0.05 M, pH 8.9, dialyzed overnight, and layered on a 1.2 × 60 cm column containing the DEAE-Sephadex equilibrated at pH 8.9. A stepwise pH gradient was established between pH 8.9 and 6.9. The effluent was monitored for pH by using a ceramic junction electrode and for optical density at 407 and 280 nm. Each of the eluting peaks was assayed as described above.

RESULTS

We were seeking any difference between the strain resistant to acriflavin (A strain) and the sensitive strain (T strain) that would help characterize how acriflavin (acr) affects *L. tarentolae*. Tables I and II summarize the results. The term "significant dyskinetoplasia" is an operational definition which refers to more than 10% dyskinetoplasia. A culture in which more than 10% of the population was dyskinetoplastic could not be subcultured in the presence of acr. Any organisms that grew out thereafter in the absence of the drug had normal kinetoplasts.

Definition of Resistance by Response to Acr

The responses of the A and T strains to different concentrations of acr are presented in Figs. 3 *a* and 3 *b*. The T strain became significantly dyskinetoplastic in the absence of red cell extract (RCE) at about 175 ng/ml acr. Growth, however, was not affected until 400 ng/ml acr. In the presence of RCE, growth was markedly affected at 100 ng/ml; dyskinetoplasia was significant by 50 ng/ml. The A strain, on the other hand, was unaffected by acr concentrations up to 1000 ng/ml unless RCE was present. In that case, it became significantly dyskinetoplastic and growth was inhibited at 200 ng/ml.

Growth Curves

The growth and dyskinetoplasia curves for both strains in the presence of 470 ng/ml acr and 20

TABLE II

Comparison of Sensitive (T) and Resistant (A) Strains of L. tarentolae

Condition	A	T
Fluorescence distribution		
+ Acriflavin, 470 ng/ml	kinetoplast only	same as A
+ Acriflavin, 470 ng/ml + red cell extract (RCE)	kinetoplast, cytoplasm and nucleus	same as A
Acriflavin-^{3}H uptake		
−RCE	progressive with growth	same as A
+RCE	2 times amount as without RCE at all times	same as A
Acriflavin-^{3}H distribution		
−RCE	mitochondrion and kinetoplast; cell surface; lipid; nucleus	same as A
+RCE	mitochondrion and kinetoplast; cell surface; lipid; some movement into nucleus	same as A
Morphological changes during division	not so stubby as T during division process	stubby by 36 hr, elongated again by 72 hr

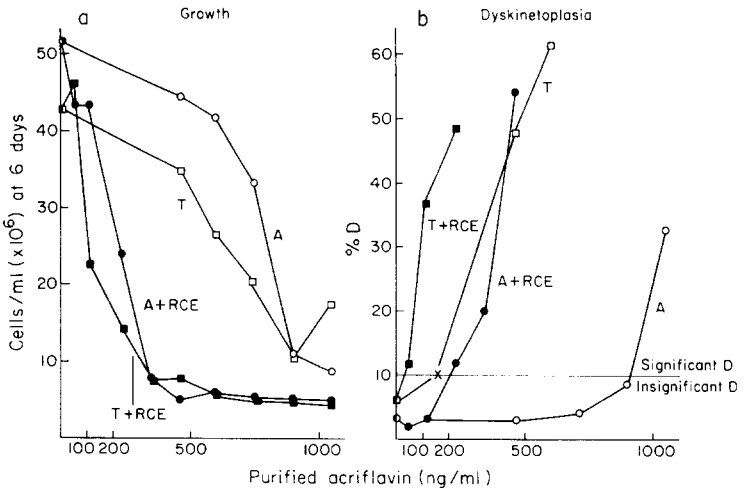


FIGURE 3 The effect of purified acr on two strains of *L. tarentolae* in the presence and absence of red cell extract (RCE). The strain sustaining exposure to acr is denoted acr-resistant (A strain). The sensitive strain is denoted as Trager's strain (T strain). (*a*) Growth of the two strains. Note that RCE inhibits growth at lower concentrations of acr and that at any given concentration (until growth is completely inhibited) A grows better than T. Each point is the average of two flasks. (*b*) Dyskinetoplasia. X denotes the concentration of acr where T generally becomes significantly dyskinetoplastic upon initial exposure. "Significant" is an operational definition, as any population with greater than 10% dyskinetoplasia cannot be subcultured in the presence of acr. RCE enhances the ability of acr to cause dyskinetoplasia in both strains. Each point is the average of two flasks.

μg/ml hemin are presented in Fig. 4. The A strain with or without acr had a slightly longer lag period and reached stationary phase about 24 hr later than the T strain without acr. Semilog plots of the same data revealed no difference in the population doubling time which was 12 hr. In the presence of acr, the T strain began to divide within 24 hr but division tapered off after 36 hr and stopped by 72 hr. Meanwhile, dyskinetoplasia remained insignificant for the A strain but was marked in the T strain within 36 hr. During log phase, T tended to become stubbier than A, which remained elongated. This characteristic was independent of the presence of acr. If RCE along with acr was added to either strain, the slightly longer lag period of A was retained but both strains became significantly dyskinetoplastic within 36 hr.

Hemin Affects the Response to Acr

Simpson (1968) had previously shown that 200 ng/ml hemin was required for growth of *L. tarentolae* in defined medium without acr. That requirement was confirmed for the A strain as well. The response of both strains to acr in the presence of different concentrations of hemin is shown in Figs. 5 *a* and 5 *b*. Both strains responded to increasing hemin concentrations with increased growth. While the A strain showed dyskinetoplasia at hemin concentrations below 10 μg/ml, at or above 20 μg/ml dyskinetoplasia was insignificant. In contrast, the T strain grew normally in the presence of 470 ng/ml acr only when the concentration of hemin was as high as 60 μg/ml. At that concentration of hemin and acr, however, T could not be subcultured: it died off and became dyskinetoplastic after four to six subcultures. When RCE was present as well as acr, the T strain never attained normal growth or insignificant dyskinetoplasia even at 80 μg/ml hemin; only at 40 μg/ml hemin did the A strain achieve normalcy.

In Figs. 5 *a* and 5 *b*, the points representing no added hemin are particularly interesting. Without additional hemin but with acr (regardless of the presence of RCE), *both strains became dyskinetoplastic without any increase in cell number.* The effect was particularly noticeable when time *versus* growth and time *versus* dyskinetoplasia were examined for the early hours after subinoculation into medium

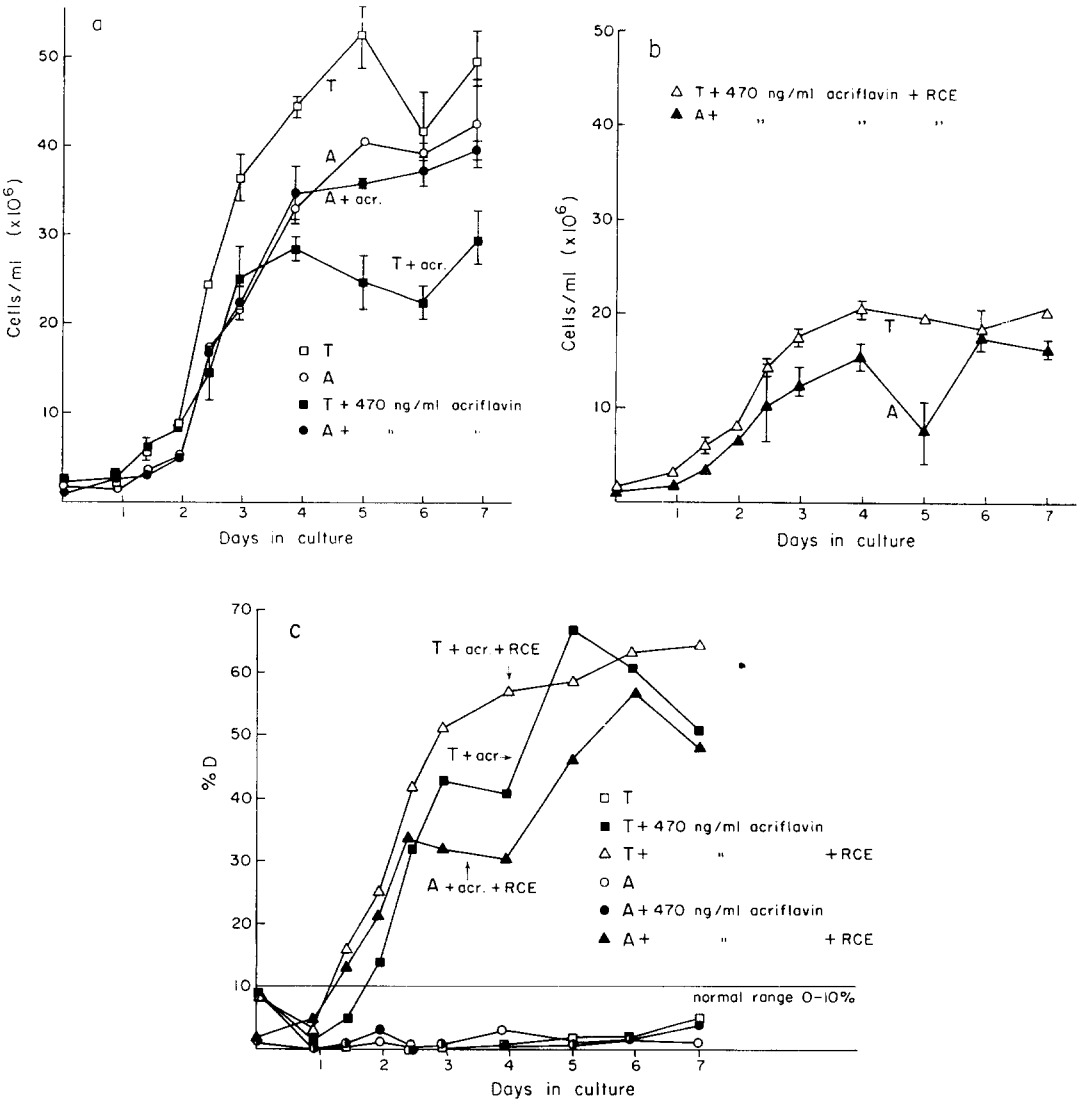


FIGURE 4 (*a*) Growth curves for T and A with and without acriflavin. Note slightly increased lag time for A ± acr before it goes into rapid-phase growth. Each point is the average of two flasks. Bars indicate ranges. (*b*) Growth curves with acriflavin and red cell extract (RCE). RCE inhibits growth of both strains. Increased lag time for A is retained. Each point is the average of two flasks. Bars indicate ranges. (*c*) Dyskinetoplasia for growth curves in Figs. 4 *a* and 4 *b*. A does not become significantly dyskinetoplastic unless RCE is present. Each point is the average of two flasks.

containing no hemin but 470 ng/ml acr. By 24 hr cells without added hemin were significantly dyskinetoplastic (Fig. 6).

Two mechanisms for the dyskinetoplastic process have been proposed: (*a*) a gradual dilution of K-DNA through division as suggested by Simpson (1968), and (*b*) a single division leaving one normal and one dyskinetoplastic cell as suggested by Cosgrove (1966). Neither mechanism seemed to explain the results described in the following experiment. Cells were inoculated into medium containing no hemin and no acr, into medium with hemin and no acr, into medium with no hemin but with acr, or into medium with both hemin and acr. Samples were taken every 4 hr up to 12 hr and every 3 hr thereafter up to 33 hr (Table III). We

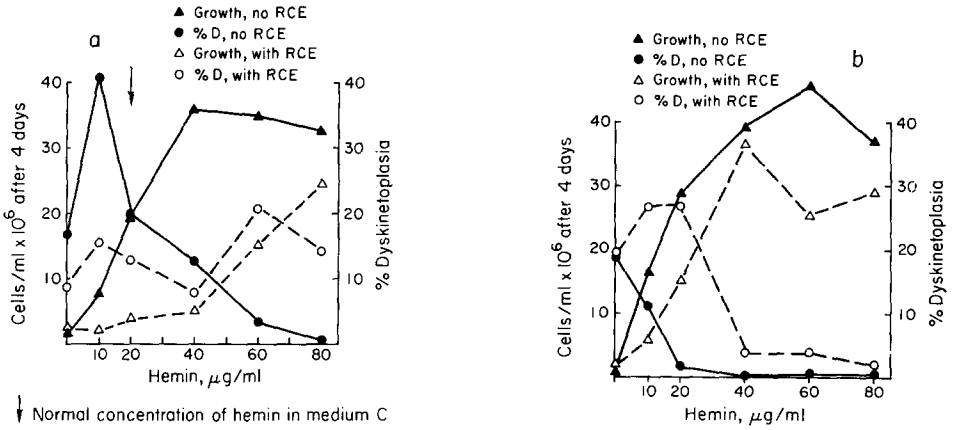


FIGURE 5 (*a*) The relationship of hemin to the acriflavin response of T. 470 ng/ml acr present in each culture. Note significant dyskinetoplasia without growth at 0 μg/ml hemin. Dyskinetoplasia at 40 μg/ml (no RCE) prevents subculture. Each point is the average of two flasks. (*b*) The relationship of hemin to the acriflavin response of A. 470 ng/ml acr present in each culture. Note significant dyskinetoplasia without growth at 0 μg/ml hemin. Compare with Fig. 5 *a*. Each point is the average of two flasks.

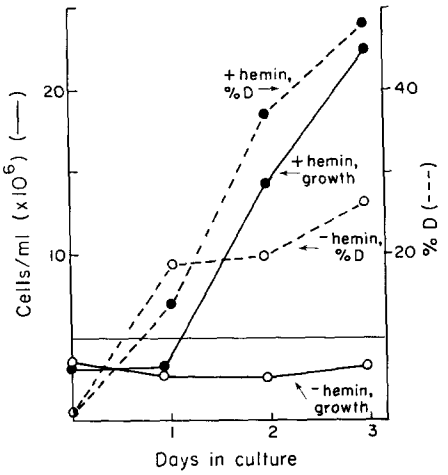


FIGURE 6 Growth and dyskinetoplasia curves for T in presence of 470 ng/ml acriflavin with and without hemin. Note the significant dyskinetoplasia within 24 hr, even though the population does not increase in number. Each point is the average of two flasks.

hypothesized that, for a single division mechanism to hold, the percentage of cells with two nuclei and one kinetoplast ($2N + 1K$) must be equal to or be greater than the percentage of dyskinetoplastic cells ($2N + 0K$ and $1N + 0K$) at a later time when dyskinetoplasia has become significant. Also, the percentage of $2N + 1K$ cells must be significantly higher in cells treated with acr than in untreated cells. In fact, the results indicated that both drug-treated and untreated populations sustained about 3–4% of their cells with $2N + 1K$. Regarding the dilution-through-division mechanism, we noted that cells grown without hemin but with acr became significantly dyskinetoplastic within 21 hr. To explain the data as a dilution phenomenon would require two cell divisions or more. (One cell division implies that the dyskinetoplastic cell arose by the all-or-none mechanism, which we have just excluded.) There was hardly time for two cell divisions within 21 hr, as the normal doubling time during log phase was 12 hr. Moreover, we would have expected that the population grown with hemin would have been more highly dyskinetoplastic, because more cells had already divided. Since this was not the case, even at 33 hr, we were unable to provide support for a mechanism involving dilution during the early stages of a culture's becoming dyskinetoplastic. Since in all cases there were 7–15% dividing forms, we could not exclude the necessity for division as dyskinetoplasia progressed.

Fluorescence in the Absence of Hemin

Previously (Strauss, 1971 *b*) it was shown that under normal growth conditions acr fluorescence was concentrated in the kinetoplast of both strains unless RCE was present. In that case, fluorescence appeared in the nucleus and cytoplasm as well. Moreover, the fluorescence of dye in cells

TABLE III

*Development of Dyskinetoplasia in T in the Presence and Absence of 20 μg/ml Hemin (with or without 470 ng/ml Acriflavin)**

Condition	Time	Dyskinetoplastic cells	Per cent cells: Dividing forms (DF)				Cells/ml
		(1 N + 0 K) + (2 N + 0 K)	2 N + 1 K	2 N + 2 K	1 N + 2 K	Total DF	
	hr						× 10^6
+Hemin, −acr	0						1.62
	4	7	2	2	1	5	1.96
	8	5	4	2	4	10	1.46
	12	4	3	4	2	9	1.84
	15	3	4	6	2	12	1.60
	18	5	2	6	1	9	2.26
	21	5	2	5	2	9	2.32
	24.3	6	3	3	2	8	1.84
	27	5	5	5	3	13	2.64
	30	1	2	8	1	11	3.74
	33	2	3	9	4	16	4.16
		Average: 3.0					
−Hemin, −acr	0						1.74
	4	9	2	1	1	4	1.38
	8	8	2	1	1	4	1.04
	12	7	3	3	1	7	1.12
	15	10	3	2	3	8	1.26
	18	5	3	4	2	9	1.52
	21	8	8	5	2	15	1.88
	24.3	3	2	3	3	8	2.42
	27	6	2	6	3	11	2.74
	30	6	4	4	6	14	3.12
	33	5	2	2	2	6	3.72
		Average: 3.1					
+Hemin, +acr	0						1.56
	4	7	2	2	2	6	1.74
	8	7	3	4	2	9	—
	12	5	6	1	2	9	1.92
	15	11	4	2	2	8	1.66
	18	7	4	1	3	8	1.52
	21	11	2	5	1	8	2.30
	24.3	12	2	2	1	6	2.48
	27	18	5	6	3	14	2.66
	30	10	6	6	2	14	3.04
	33	18	4	3	1	8	3.26
		Average: 3.8					
−Hemin, +acr	0						1.68
	4	8	3	2	3	8	1.52
	8	9	2	1	2	5	1.40
	12	12	3	2	2	7	1.14
	15	5	3	6	5	14	1.08
	18	12	3	1	2	6	1.20
	21	13	3	2	1	6	1.36
	24.3	13	3	1	2	3	—
	27	16	2	3	4	9	1.24
	30	16	5	3	1	9	1.82
	33	20	6	3	4	12	1.76
		Average: 3.3					

* Each figure is the average of two flasks.

incubated in high concentrations of acr for several hours in the absence of hemin was diffuse (Simpson, 1968). The addition of hemin immediately caused the diffuse fluorescence to localize in the kinetoplast region.

Grown for 48 hr without hemin but with 470 ng/ml acr, the sensitive strain exhibited two types of fluorescence, the usual yellow-green and a brilliant orange. The cytoplasm was often pale yellow-green; occasionally the nucleus was outlined against the cytoplasm with a somewhat greater intensity. One or more orange bodies, round or elongated, and one or more yellow-green bodies fluoresced brilliantly. When only one body was present, it was either yellow-green or orange and located in the kinetoplast region. The orange fluorescence appeared to fade the more quickly, although both were short-lived. Orange fluorescence in the resistant strain under similar conditions was less marked. Since Giemsa-stained preparations from the same experiments showed few cells with increased granule populations, it was unlikely that the fluorescent particles were similar to volutin granules (Ormerod, 1961) induced in trypanosomes by various drug treatments. Cells without hemin or acr did not fluoresce. Therefore, it was unlikely that low hemin induced the cells to synthesize porphyrin, which would have fluoresced orange, if iron were not associated with the porphyrin ring (Udenfriend, 1962, 1969). One of the principal porphyrins found in *L. tarentolae* is uroporphyrin (Gaughan and Krassner, 1971). Uroporphyrin generally fluoresces red, although Konstantinova-Schlessinger (1965) reports a uroporphyrin b with orange or orange-red fluorescence. Hence, the orange fluorescence may be due to increased degradation of hemin to the noniron-containing uroporphyrin.

Arginine

Another nutritional requirement studied for its possible effect on the dyskinetoplastic process was arginine, reportedly involved in yeast cytochrome synthesis (Wilkie, 1970). A and T strains differed significantly in their requirement for arginine (Fig. 7). 5 μg/ml (23 μM) permitted 50% continuous growth of T; 10 μg/ml permitted maximal growth. 12.5 μg/ml arginine (58 μM) allowed 50% continuous growth of A; 40 μg/ml allowed maximal continuous growth. The presence of acr prevented growth of A beyond the first culture at concentrations of arginine lower than 20 μg/ml (92 μM). The normal concentration of arginine in medium C is 300 μg/ml.

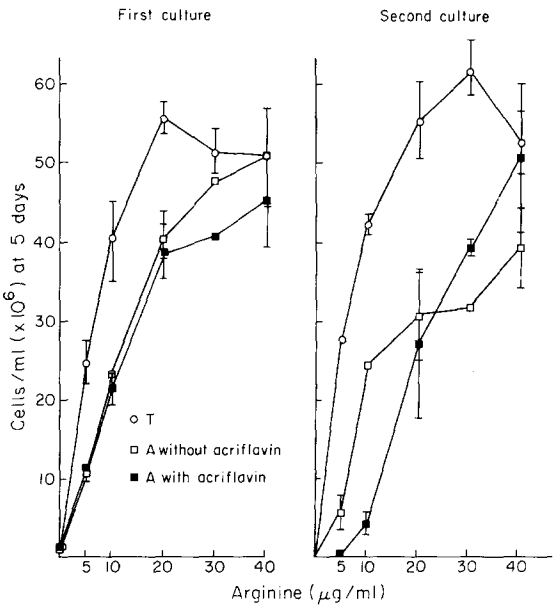


FIGURE 7 Arginine requirement of A without acriflavin, A with 470 ng/ml acriflavin, and T. Note the greater arginine requirement of A for continuous growth. Each point is the average of two flasks. Bars indicate ranges.

Fine Structure

The fine structure of *L. tarentolae* was described by Trager and Rudzinska (1964) and by Strauss (1971 *a*). Of particular interest here were the mitochondrion and kinetoplast (Figs. 8–12). In both strains grown without acr, mitochondria were round or elongated, the cristae stubby or finger-like and the matrix sometimes electron-opaque. The nucleoid appeared with a diameter of 90–95 nm and a length of up to 2.0 μ. In the presence of 470 ng/ml acr, characteristic changes occurred in the mitochondria of both strains, although not all the mitochondrial profiles of either strain were affected and fewer of the A strain were affected than of the T strain. As described in *L. tarentolae* (Trager and Rudzinska, 1964) and in *Saccharomyces cerevisiae* (Yotsuyanagi, 1962 *a*, *b*), cristae were dilated or elongated until they appeared as long strands (Figs. 9 and 10). In the T strain but not in the A strain, the nucleoid became first disordered, then diminished, and finally appeared as a smudge. If RCE was added to either strain along with acr, cristae abnormalities were enhanced. The nucleoid of *both* strains became disarrayed ond finally remained only as a smudge (Figs. 11–12).

Acriflavin Metabolism

The availability of tritiated acr made possible tracer studies to characterize and compare the handling of the drug by the two strains. The amount of the dye present in both strains grown with different amounts of hemin is shown in Table IV. The presence of hemin in the medium sharply reduced the amount of tritiated dye found with cells after they had grown in its presence for 5 days. Increasing the concentration of hemin beyond 20 μg/ml, the usual concentration of hemin in medium C, tended to further reduce the uptake by the cells. Thus, the data support Simpson's observations (1968) that cells retained less acr when hemin was present. At 20 μg/ml, however, both strains retained approximately the same amount of dye, again confirming that resistance on the part of the A strain was not due to impaired permeability to acr. Previously it had been shown that the presence of RCE or either equine or bovine hemoglobin doubled the uptake of the dye in both strains (Strauss, 1971 *b*) but that merely doubling the concentration of dye taken up by the A strain did not make the strain sensitive.

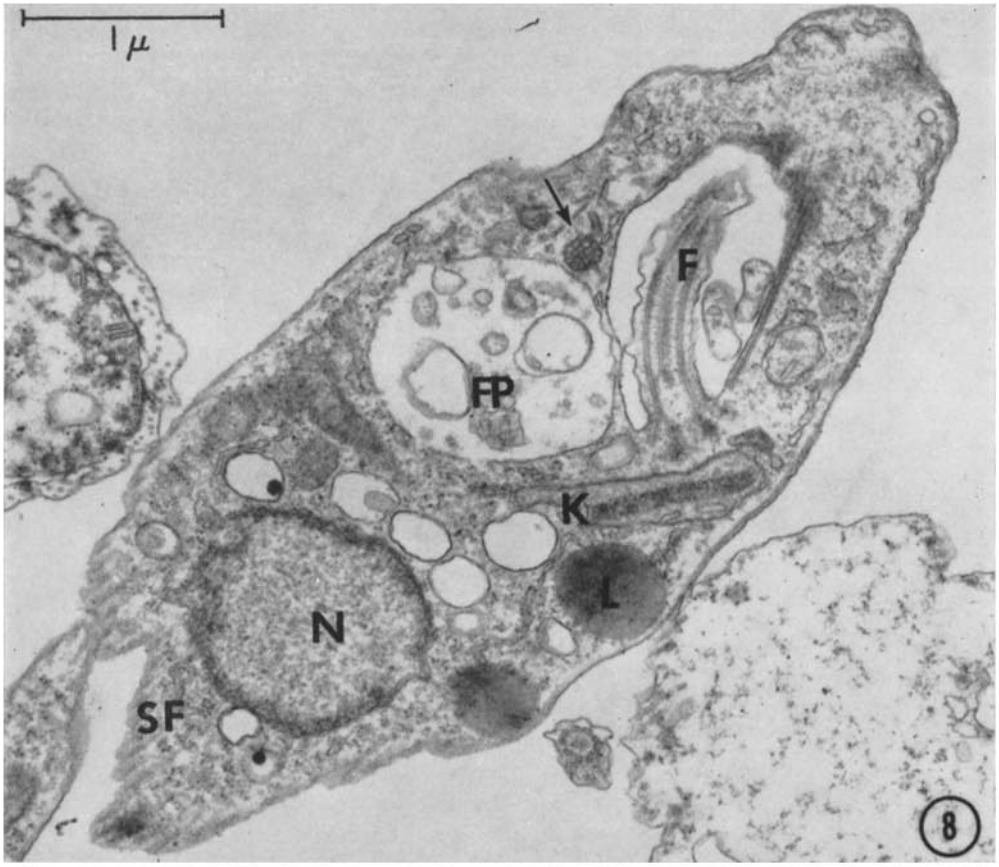
The results of butanol/acetic acid paper chromatography of sarkosyl lysates prepared under similar conditions are presented in Table V. The lysates were also chromatographed in 3% NH_4Cl at the same time; those results served to confirm the data presented in the table. Extracts prepared from cells grown 5 days with the dye had two major components and sometimes a minor one. The largest peak had an R_f of 0.63, the same as the R_f of the tritiated dye and of the purified acr standard. The second peak had an R_f of less than 0.05. The minor component had an R_f of 0.30. Neither the second peak nor the minor component could be explained as random binding of cellular components to the dye, because extracts prepared from cellular material mixed with isotope or cells incubated with isotope for 3 hr had none of the minor peak and less than 5% of the component found at the origin. Adding RCE to or removing hemin from the medium in which the cells were grown greatly increased the amount of freely moving acr. A specific detoxification mechanism seems possible, one interfered with by RCE and enhanced by hemin.

Electron Microscope Radioautography

As noted previously, the only direct evidence for intercalation of acr with DNA occurring in vivo is the fluorescence localization in hemoflagellates (Simpson, 1968; Strauss, 1971 *b*). In order to substantiate the fluorescence observations, it was decided to perform electron microscope radio-

FIGURE 8 *L. tarentolae* T strain in the absence of acr. A typical parasite is shown in cross-section. The arrow indicates a structure containing numerous small vesicles. *F*, flagellum; *FP*, flagellar pocket; *K*, kinetoplast containing ordered nucleoid; *L*, lipid droplets; *N*, nucleus; *SF*, subpellicular fibers. Electron micrograph. × 26,000.

FIGURE 9 *L. tarentolae* A strain in the presence of 470 ng/ml acr. Note the abnormal, stringy cristae (*) and the normal mitochondrial profile (*M*) in the same cell. *L*, lipid droplet; *F*, flagellum; *K*, kinetoplast containing ordered nucleoid. Electron micrograph. × 41,000.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F
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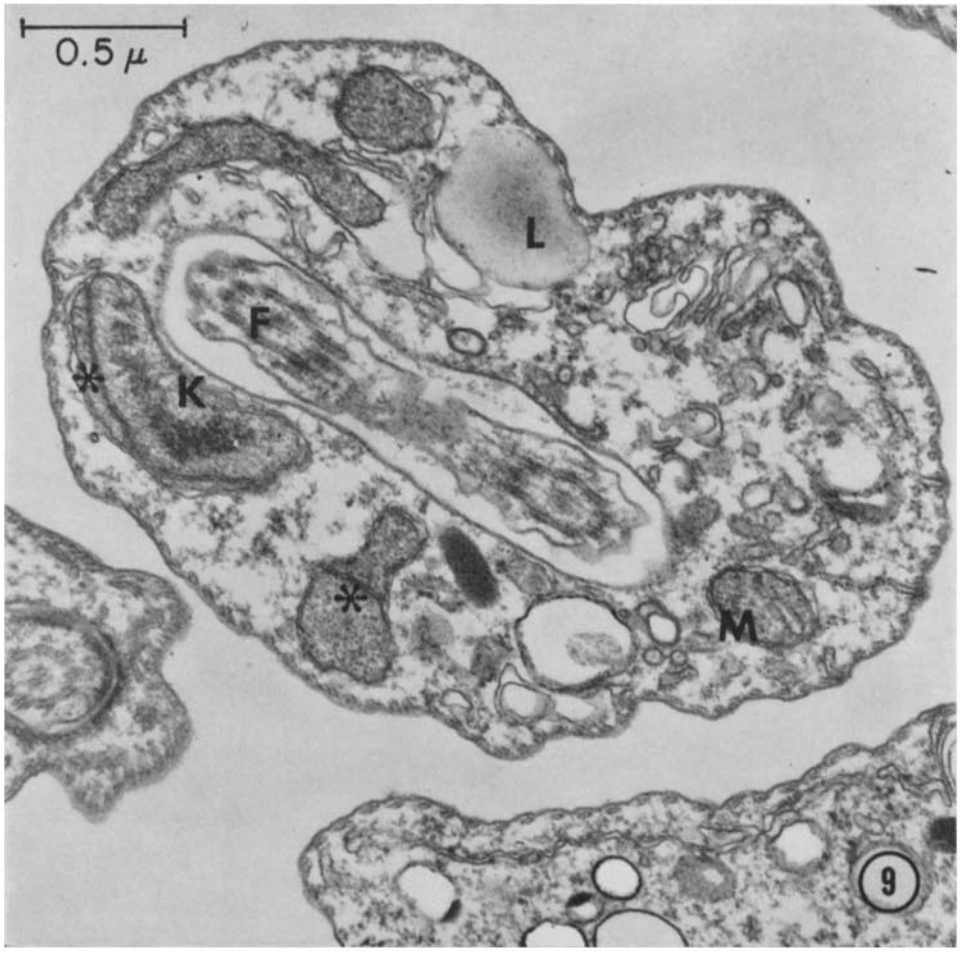
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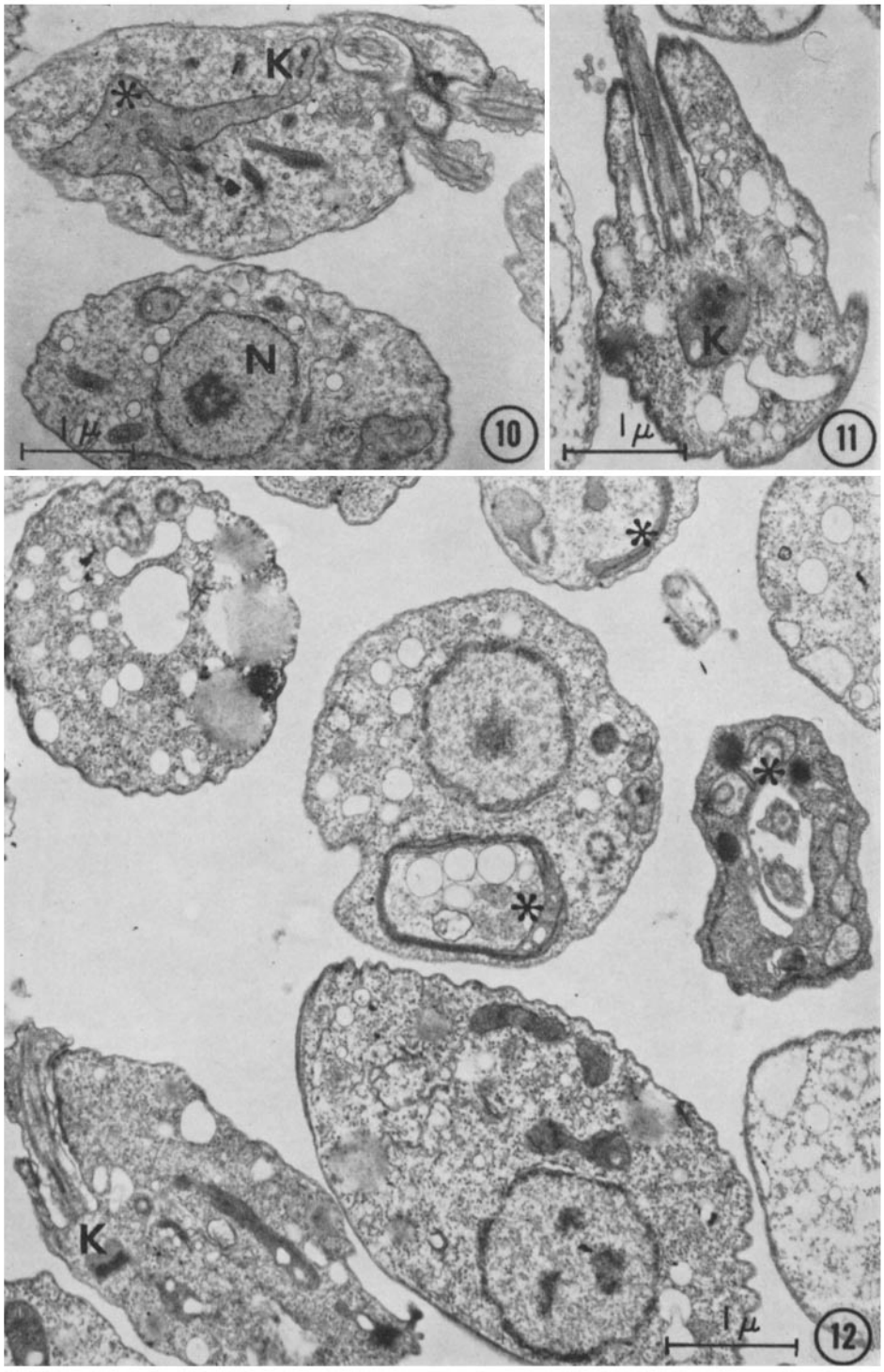

FIGURE 10 T strain in presence of 470 ng/ml acr. The mitochondrion of this dividing cell contains numerous swollen cristae (*). The kinetoplast (*K*) appears disjoint. *N*, nucleus. Electron micrograph. × 15,300.

FIGURE 11 T strain in presence of 470 ng/ml acr and RCE. The nucleoid of the kinetoplast (*K*) appears as a smudge. Electron micrograph. × 17,000.

FIGURE 12 A strain grown 5 days with 470 ng/ml acr and RCE. Mitochondria are now abnormal (*) and the kinetoplast (*K*) is disordered. Electron micrograph. × 18,000.

TABLE IV

*Effect of Hemin on Acriflavin Uptake**

Strain	Hemin concentration	cpm/10^6 cells
	μg/ml	
T	0	6268
	0	6579
	20	189
	20	196
	40	140
	40	134
	60	110
	60	128
A	0	7693
	0	6650
	20	171
	20	156
	40	097
	40	079
	60	102
	60	082

* Cells were grown for 5 days in the presence of acriflavin-^{3}H and the specified amount of hemin. They were washed 5 times with SBG. Cell density was determined on the fifth wash. The pellets were dissolved in 0.5 ml Protosol, and 0.1 ml duplicate portions were counted.

TABLE V

Ascending Chromatography of Sarkosyl Lysates from Cells Grown with Acriflavin-^{3}H

Condition	Hemin	Red cell extract	Peak (R_f) in butanol/acetic acid (percentage)		
			<0.05	0.30	0.63
	μg/ml				
Acr only			—	—	100
Acr + 2% sarkosyl			3	—	97
Acr + cells lysed in sarkosyl			5	—	95
Cells incubated 3 hr in acr-^{3}H			6	—	94
T grown 5 days with acr-^{3}H	0	—	15	3	82
	0	—	24	—	76
	20	—	40	—	60
	20	—	44	—	56
	40	—	32	—	68
	60	—	33	—	67
	20	+	13	—	87
A grown 5 days with acr-^{3}H	0	—	19	—	81
	0	—	13	—	87
	20	—	30	8	62
	20	—	44	—	56
	20	—	39	—	61
	40	—	37	10	53
	40	—	45	6	49
	20	+	17	—	83

autography. Six experimental situations were examined: (1) T + 500 ng/ml acr, 2 days' growth. (2) A + 500 ng/ml acr, 2 days' growth. (3) T + 500 ng/ml acr + RCE, 2 days' growth. (4) A + 500 ng/ml acr + RCE, 2 days' growth. (5) T + 500 ng/ml acr, 5 days' growth. (6) T + 500 ng/ml acr + RCE, 5 days' growth. Fluorescence studies had shown that acr was located in the kinetoplast region in conditions (1) and (2); in the kinetoplast region, the cytoplasm, and the nucleus in conditions (3) and (4); in the kinetoplast region, if present, in condition (5); and in the nucleus, the cytoplasm, and a few kinetoplast areas in condition (6). Giemsa-stained slides had indicated that in condition (2) there was insignificant dyskinetoplasia, that in conditions (1), (3), and (4) dyskinetoplasia was significant but only partly developed, and that in conditions (5) and (6) there was maximal dyskinetoplasia. Results are tabulated in Tables VI–VIII. For raw data, see Tables IX, X.

Certain auxiliary data are available from Table VI which presents the volume data. For instance, the relative volumes occupied by the various organelles in A and T were similar under any experimental condition where both strains were examined. Also, neither acr nor acr + RCE caused significant shifts in volume distribution. (Compare A at 5 days with the other data.) In addition, both A and T strains accumulated lipid droplets between days 2 and 5 regardless of the presence of acr.

For analysis of grain distributions, the 70% probability radius was chosen, because the most desirable radius for estimating all possible sources of a given grain, the 95% probability radius, was large in comparison to an average cross-section of *L. tarentolae* (Fig. 13). Use of the latter would have yielded a distribution of all organelles rather than a distribution of grain-associated organelles. Often there were several structures found within the 70% probability radius (Fig. 13). Each structure

TABLE VI

*Volume Ratios for L. tarentolae under Different Experimental Conditions**

Experimental condition‡	% of volume distribution in each category						
	Nucleus a = (2)/(1)§	Total cytoplasm b = (3)/(1)	Mitochondrion c = (4)/(1)	Kinetoplast d = (5)/(1)	Flagellar pocket e = (6)/(1)	Lipid droplets f = (7)/(1)	Cytoplasm minus organelles $g = b - (c + d + e + f)$
T+acr, 2 days	8.3	89.1	6.9	0.50	3.8	0.17	77.7
A+acr, 2 days	9.4	89.2	7.4	0.42	3.0	0.34	78.0
T+acr+RCE, 2 days	9.7	87.0	6.5	0.42	2.5	0.28	77.3
A+acr+RCE, 2 days	8.6	90.7	6.7	0.19	2.6	0.47	80.7
T, no acr, 5 days	6.2	95.0	7.9	0.77	3.8	2.1	80.4
T+acr, 5 days	10.9	84.3	7.2	0.41	6.5	1.9	68.3
A+acr, 5 days	8.6	90.7	6.7	0.42	2.9	2.6	78.1
T+acr+RCE, 5 days	13.5	91.8	6.5	0.34	2.2	3.3	79.5

*By the method of Weibel et al., 1966.

‡ T, sensitive strain; A, resistant strain; acr, purified, tritiated acriflavin, 500 ng/ml; RCE, red cell extract.

§ Figures derived from Table IX.

TABLE VII

*Per Cent Distribution of Grains Associated within the 70% Probability Radius with the Given Category**

Experimental condition‡	% of distribution of grains with the given category							
			Distribution of cytoplasmic grains					
	Nucleus (3)/(2)§	Cytoplasm (4)/(2)	Surface (5)/(4)	Mitochondrion (6)/(4)	Kinetoplast (7)/(4)	Flagellar pocket (8)/(4)	Lipid droplets (9)/(4)	Cytoplasm only (10)/(4)
T+acr, 2 days	4.2	99.8	58.5	12.6	0.2	5.8	0.6	21.0
A+acr, 2 days	7.0	99.1	57.3	6.7	0.8	6.5	2.7	21.3
T+acr + RCE, 2 days	9.0	98.8	53.8	7.6	0.5	4.9	1.5	23.2
A+acr + RCE, 2 days	9.4	99.0	58.9	9.1	0.4	4.4	1.3	22.1
T+acr, 5 days	8.1	99.7	65.2	21.6	1.8	2.1	9.6	20.0
T+acr + RCE, 5 days	13.9	97.9	58.4	27.2	0.6	4.0	21.2	9.8

* By the method of Salpeter et al., 1969.

‡ T, sensitive strain; A, resistant strain; acr, purified, tritiated acriflavin, 500 ng/ml; RCE, red cell extract.

§ Figures derived from Table X.

was scored, while the absolute number of grains counted was also tallied. Therefore, the sum of grains associated with different structures (compartments) listed in Table X exceeds the absolute number of grains counted. Percentages in Table VII refer to the number of grains associated with a given compartment divided by the absolute number of grains that were cell associated. The latter represented roughly 90% of all grains counted.

Regarding the final calculations in Table VIII, the figures refer to per cent grains associated with a given compartment (within the 70% probability radius) divided by the per cent volume occupied by that compartment. No attempt was made to normalize the grains that were surface associated. These represented about 60% of all grains counted.

From the data in Table VIII the following conclusions can be drawn: (*a*) the grains were nonrandomly distributed as shown by the low association of grains with cytoplasm. (*b*) The grains seemed to be distributed in a similar fashion for both A and T under any given experimental situation. (*c*) The grains seemed to be preferentially associated with mitochondrion and kinetoplast, but not with the one more than with the other. (*d*)

TABLE VIII

Grain Distribution of Acriflavin-^3H—Final Calculations (Per Cent Grains Associated with a Given Category/Per Cent Volume Occupied by that Category)

Experimental condition*	Category					
	Nucleus	Mitochondrion	Kinetoplast	Flagellar pocket	Lipid droplets	Cytoplasm only
T+acr, 2 days	4.2/8.3	12.6/6.9	0.2/0.5	5.8/3.8	0.6/0.17	21.0/77.73
	0.51	1.83	0.40	1.53	3.53	0.27
A+acr, 2 days	7.0/9.4	6.7/7.4	0.8/0.42	6.5/3.0	2.7/0.34	21.3/76.04
	0.74	0.91	1.90	2.17	7.94	0.28
T+acr+RCE,	9.0/9.7	7.6/6.5	0.5/0.42	4.9/2.5	1.5/0.28	23.2/77.30
2 days	0.93	1.17	1.19	1.96	5.36	0.30
A+acr+RCE,	9.4/8.6	9.1/6.7	0.4/0.19	4.4/2.6	1.3/0.47	22.1/80.74
2 days	1.09	1.36	2.11	1.69	2.78	0.27
T+acr, 5 days	8.1/10.9	21.6/7.2	1.8/0.41	2.1/6.5	9.6/1.9	20.0/68.29
	0.74	3.00	4.39	0.32	5.05	0.29
T+acr+RCE,	13.9/13.5	27.2/6.5	0.6/0.34	4.0/2.2	21.2/3.3	9.8/79.46
5 days	1.03	4.18	1.76	1.82	6.42	0.12

* T, sensitive strain; A, resistant strain; acr, purified, tritiated acriflavin, 500 ng/ml; RCE, red cell extract.

TABLE IX

Crude Data for Determination of Relative Volumes

Experimental condition*	Hits per category‡						
	Whole cell (1)	Nucleus (2)	Total cytoplasm (3)	Mitochondrion (4)	Kinetoplast (5)	Flagellar pocket (6)	Lipid droplets (7)
T+acr, 2 days	11,650	970	10,375	809	58	441	20
A+acr, 2 days	13,250	1252	11,825	974	56	394	45
T+acr+RCE, 2 days	14,925	1452	13,000	974	62	369	42
A+acr+RCE, 2 days	11,000	949	9975	736	21	290	52
T, no acr, 5 days	12,400	766	11,775	977	95	465	260
T+acr, 5 days	14,300	1552	12,050	1026	59	383	268
A+acr, 5 days	12,100	1038	10,975	810	51	353	312
T+acr+RCE, 5 days	16,475	2217	15,125	1070	56	366	549

* T, sensitive strain; A, resistant strain; acr, purified, tritiated acriflavin, 500 ng/ml; RCE, red cell extract.

‡ By the method of Weibel et al., 1966.

The grains seemed to be markedly associated with those structures which appeared to be lipid droplets. (*e*) In the presence of RCE there were slightly more grains associated with nuclei than in the absence of RCE. (*f*) Association with kinetoplast-mitochondrion and nuclear compartments increased between 2 and 5 days.

In short, the EM radioautography data do not support the contention that acr is strictly localized in the kinetoplast of *L. tarentolae*. Rather, they indicate that intracellular acr is preferentially associated with the mitochondrion-kinetoplast complex as a whole and with those structures that appear to be lipid droplets. That the fluorescence of the dye is localized in the kinetoplast, however, can still be interpreted as evidence for interaction of acr with K-DNA.

Drug Treatments

Several drugs were administered to both strains for their ability to interfere with mitochondrial metabolism or as a comparison of mitochondrial metabolism. They included ethidium bromide (EB), cycloheximide (CH), and chloramphenicol (CAP). (See Tables I and II.)

TABLE X

*Crude Grain Count Data: Grains Associated within the 70% Probability Radius with the Given Category**

Experimental condition‡	Total grains counted (1)	Total grains cell associated (2)	Grains associated with nuclei (3)	Grains associated with cytoplasm (4)	Cytoplasmic grains: Surface grains (5)	Mitochondrial grains (6)	Kinetoplast grains (7)	Flagellar pocket grains (8)	Lipid droplet grains (9)	Cytoplasm only grains (10)
	Total grains associated with different categories									
T+acr, 2 days	1741	1580	66	1577	922	198	3	91	9	331
A+acr, 2 days	1691	1529	107	1515	868	102	12	98	41	322
T+acr+RCE, 2 days	2133	1996	179	1973	1062	151	10	97	30	457
A+acr+RCE, 2 days	1271	1151	108	1139	671	104	4	50	15	252
T+acr, 5 days	1511	1376	112	1372	895	296	25	29	132	275
T+acr+RCE, 5 days	2002	1942	270	1902	1111	517	11	77	403	187

* By the method of Salpeter et al., 1969.

‡ T, sensitive strain; A, resistant strain; acr, purified, tritiated acriflavin, 500 ng/ml; RCE, red cell extract.

EB: The A strain showed twice the resistance to EB shown by the T strain (Fig. 14). From a single culture which grew continuously at 160 ng/ml EB, a strain (B strain) was developed that was resistant both to 470 ng/ml acr and to 260 ng/ml EB separately but not together. Resistance to EB remained fast for 2.5 months in the absence of the drug. When that resistance was lost, the organisms had still retained resistance to 470 ng/ml acr. The tolerance of the B strain could be raised to 480 ng/ml EB, if the amount of hemin in medium C were doubled to 40 μg/ml. Thus, hemin played a role in the mode of action of a second agent known to cause dyskinetoplasia in hemoflagellates (Newton, 1957, 1964; Steinert, 1969; Steinert et al., 1969). From the B strain, cultures were obtained that were resistant both to 470 ng/ml acr and 260 ng/ml EB together (A–B strain). The double resistance, however, was not fast.

CAP AND CH: Both A and T strains tolerated 500–1000 μg/ml CAP in the absence of acr and 500 μg/ml CAP in the presence of acr. The strains responded differently, however, to CH, T tolerating 2.24 ng/ml and A tolerating 0.56 ng/ml CH in the absence of acr. CH and CAP neither interfered with dyskinetoplasia attained by T in the presence of 470 ng/ml nor promoted the dyskinetoplasia of A, when A was grown with acr. While the differential sensitivity to CH may be entirely unrelated to acr resistance, it is possible that a process affected by CH may be one of the changes involved in the resistance to acr.

Acriflavin-Sensitizing Factor

Red cell extract or hemoglobin at 37 μg/ml could supply the parasite with the required hemin. But when bovine or equine RCE or 37 μg/ml equine hemoglobin obtained from Pentex Biochemical (Hb-P) was added to either strain in the presence of acr, both strains became dyskinetoplastic and could not be subcultured (Strauss, 1971 *b*; see Fig. 15). With RCE or Hb-P in the medium, acr fluorescence appeared in the nucleus and cytoplasm as well as in the kinetoplast of both strains. Since 37 μg/ml bovine hemoglobin (Hb-S) did not enhance the sensitivity of the A strain to acr, even though it also doubled the uptake of acr-^{3}H, it was suggested that the substance affecting the response to acr was not hemoglobin but some minor component of red cells (Strauss, 1971 *b*).

Solution of Hb-P in 0.02 N acetic acid did not alter its activity, nor did heating at 56°C for 14 hr. Boiling for 20 min partly reduced the activity. Dialysis of Hb-P against four changes of saline over a period of 5 days did not eliminate the factor. Chromatography of Hb-P through Sephadex in saline; 0.05 M Tris, pH 8.6; or 0.05 M Tris, pH 8.6–saline did not separate the activity from the hemo-

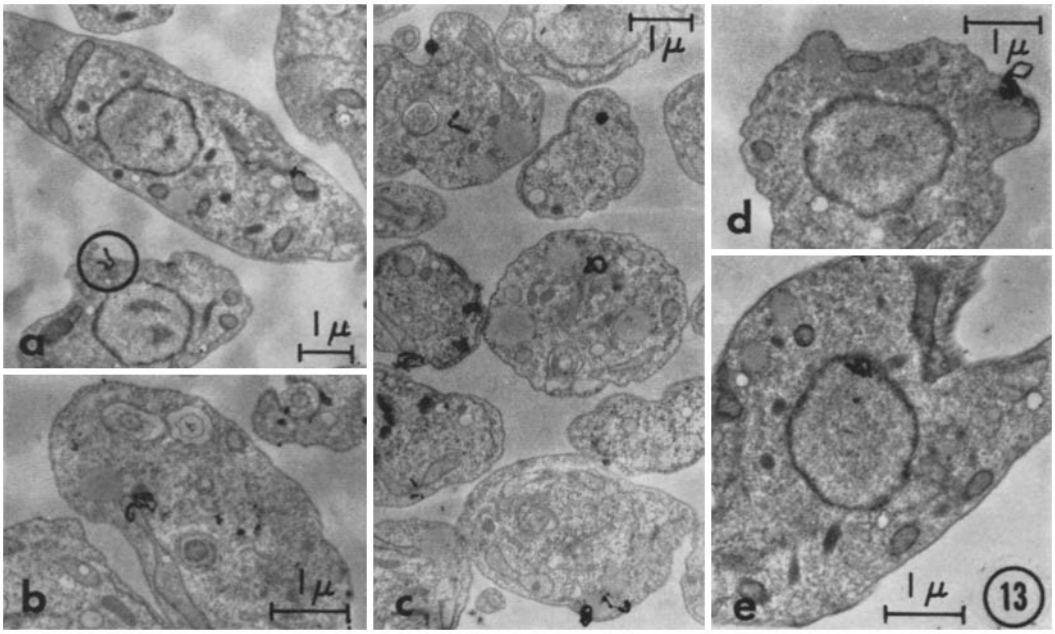

FIGURE 13 Electron microscope radioautography. Sections stained before dipping. (*a*) T + 500 ng/ml acr, 5 days. Circle represents 70% probability radius. × 6900. (*b*) T + 500 ng/ml acr, 5 days. × 10,100. (*c*) T + 500 ng/ml acr, 5 days. × 8700. (*d*) T + 500 ng/ml acr + RCE, 5 days. × 9600. (*e*) T + 500 ng/ml acr + RCE, 5 days. × 10,200.

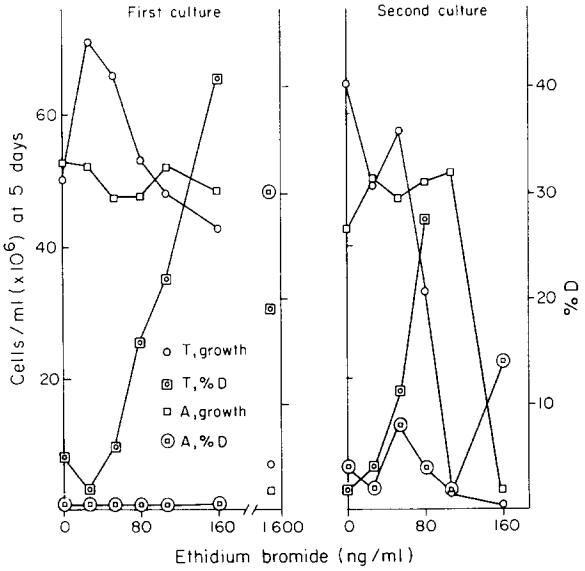

FIGURE 14 Effect of ethidium bromide on growth and dyskinetoplasia of resistant (A) and sensitive (T) strains. Note relative resistance of A strain. Individual flasks from first culture were subcultured (second culture) to determine effect of continuous exposure to ethidium bromide. Each point is the average of two flasks.

globin peak (Fig. 16). The trailing peak, which comprised less than 0.1% of the material on the basis of absorption at 280 nm, contained no detectable activity in the amounts recovered by this method.

In an attempt to remove activity from Hb-P by charge, Hb-P was eluted from a column packed with DEAE-Sephadex A50 in 0.05 M Tris buffer by descending pH gradient (Fig. 17). Peak 1 (0.025 OD280 units/ml) had variable activity. Peak 1 at the same concentration along with 33 μg/ml Hb-S had strong activity. Peak 1 caused fluorescence in the A strain to appear in the nucleus. Peaks 2–6 had no activity at concentrations between 350 and 500 μg/ml and did not cause fluorescence to appear in the nuclei of A strain cells. Combinations of nonactive, heme-containing peaks 2–6 with peak 1 produced variable activity.

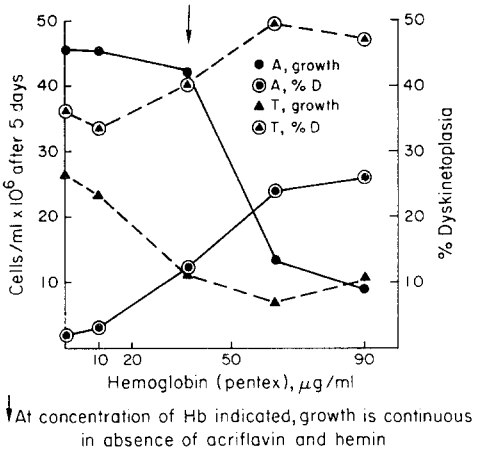

FIGURE 15 Effect of hemoglobin (Pentex) on growth and dyskinetoplasia of A and T. At 37 μg/ml, the concentration of hemoglobin which sustains growth in the absence of acr, Pentex hemoglobin raises the dyskinetoplasia of the A strain to the significant level. Growth falls off only at higher concentrations of the protein. The T strain is consistently dyskinetoplastic, but its growth is diminished by higher concentrations of hemoglobin-Pentex.

Peak 1 was concentrated by solid sucrose and rerun on Sephadex G100 in 0.05 M Tris, pH 8.6. It eluted between fractions 57 and 62, where the minor, nonheme-containing peak was observed earlier from Sephadex preparations, and it retained its activity on an OD280 basis. On the same column, cytochrome C eluted with fractions 38–44. Therefore, the material had a molecular weight less than 12.6×10^3 daltons or else was retained by Sephadex. Peak 1 had no absorbance at 407 or 450 nm, the characteristic absorption wavelengths for porphyrins, and its 260:280 ratio was 1. Hence, it was unlikely that the factor was a porphyrin. Factor activity of peak 1 was not diminished by lowering the pH to 2.0 for 24 hr at 37°C or by heating at 56°C for 24 hr, and was only partly decreased by boiling for 20 min. Reduced and oxidized glutathione, cystine and cysteine, and 2,3-diphosphoglyceric acid, all compounds found in significant amounts in red cells, were without activity.

DISCUSSION

The results presented here bear on two related problems: the mechanism of resistance to purified acr and the mode of action of acr in producing dyskinetoplasia in hemoflagellates.

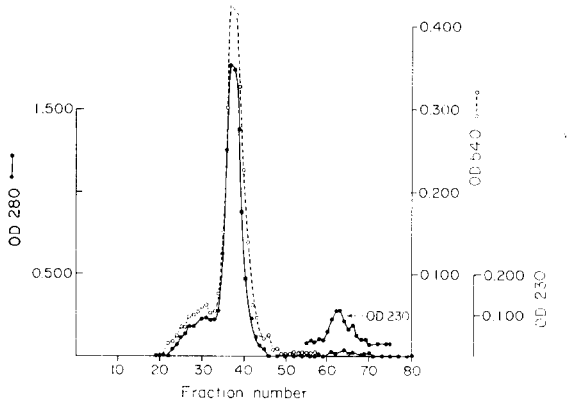

FIGURE 16 Elution plot of hemoglobin-Pentex. Gel: Sephadex G-100; solvent: saline. 5-ml fractions were collected. Note minor component eluting between fractions 60 and 70.

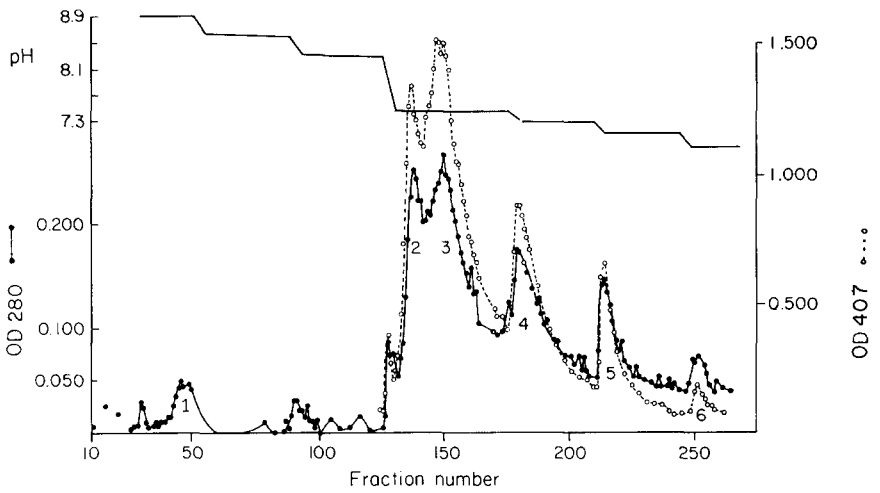

FIGURE 17 Elution plot of hemoglobin-Pentex. Gel: DEAE-Sephadex, A50; solvent: Tris, 0.05 M, pH as indicated. 3-ml fractions collected. Peak 1 had activity which was enhanced by the addition of nonactive hemoglobin-Sigma.

The experiments with tritiated acr support the earlier conclusion (Strauss, 1971 *b*) that strain A of *L. tarentolae*, which is resistant to acr, does not differ from the susceptible strain in its uptake and compartmentalization of the dye. Furthermore, hemin and the factor from red cell extract (or from hemoglobin Pentex) affect both strains in the same way, the former decreasing and the latter increasing the sensitivity to acr. The strains differ, however, in their arginine requirements and their responses to cycloheximide. The resistant strain requires more arginine for continuous growth than the sensitive strain. Since arginine is involved in mitochondrial synthesis in yeast (Wilkie, 1970), the arginine requirement of *L. tarentolae* deserves further study in relation to its possible bearing on the effects of acr on the kinetoplast and the mitochondrion. Similarly, the lower tolerance of the A strain for cycloheximide might indicate some dependence of the resistance on one or more of the several cellular functions that are inhibited by cycloheximide-cytoplasmic protein synthesis (Lamb et al., 1968), cell wall (or perhaps, in the case of *L. tarentolae*, cell surface?) synthesis (Elorza and Sentendreu, 1969), or phospholipid synthesis (Bishop and Smillie, 1970). It is interesting that the two strains do not differ significantly in their response to chloramphenicol, an inhibitor of mitochondrial protein synthesis (Lamb et al., 1968). As might perhaps have been expected, strain A is more resistant to ethidium bromide, another dye that produces dyskinetoplasia in hemoflagellates (Newton, 1957, 1964; Steinert, 1969).

McIlwain (1941) demonstrated that adenine interferes with acr's effects in bacteria. In *L. tarentolae* neither adenine nor guanine interferes with dyskinetoplasia in the strain sensitive to acr, even though both purines stimulate population growth two to three times (unpublished results). Moreover, bongkrekic acid,[3] a nonreversible inhibitor of mitochondrial adenine nucleotide translocase (Henderson and Lardy, 1970), stops population growth in both strains but does not enhance or interfere with the dyskinetoplastic process (unpublished results).

Since dyskinetoplasia in hemoflagellates grown in vitro has been associated not only with the loss of K-DNA but also with the inhibition of K-DNA synthesis (Simpson, 1968) and of synthesis of cytochromes a + a_3 and b (Hill and Anderson, 1969, 1970), and since acr seemed to localize in the kinetoplast (as seen by fluorescence microscopy), it was reasonable to suppose (Guttman and Eisenman, 1965) that the effects were a direct result of the known property of acr to intercalate with DNA (Lerman, 1961, 1963). K-DNA synthesis would then be stopped while all other cytoplasmic and nuclear processes continued, and after a few cell divisions the K-DNA would be lost. These assump-

[3] Bongkrekic acid was kindly supplied by Professor W. Berends.

tions, simple and straightforward as they seemed, have been put into question by the finding that dyskinetoplastic blood-stream trypanosomes (which are viable in host animals but cannot grow in culture) do contain K-DNA in nearly normal amounts, even though it is not visible by either light or electron microscopy (Renger and Wolstenholme, 1971; Stuart, 1971). One must assume that the K-DNA is dispersed throughout the mitochondrion, more in the manner typical of most cells other than hemoflagellates. The necessity for hemoflagellates, under most conditions, to retain condensed K-DNA might account for their 10-fold greater sensitivity to acr than, for instance, *Neurospora crassa* (Lizardi, 1971), *S. cerevisiae* (Nagao and Sugimura, 1965; Fukuhara and Kujawa, 1970), or Chang liver cells (Bose et al., 1966).

Dispersal could account for other observations presented here, for instance, the low levels of dyskinetoplasia in normal, untreated populations, and for significant dyskinetoplasia in apparently nondividing populations such as those obtained in the presence of acr and the absence of hemin (Table III). It could also account for the inability of cells treated with acr or EB to incorporate thymidine-^{3}H into the nucleoid (Steinert and Van Assel, 1967; Hill and Anderson, 1969; Steinert, 1969; Steinert et al., 1969; Riou, 1970).

In any case it seems clear that the action of acriflavin is more complex than previously supposed. At least two observations suggest that components in addition to kinetoplast-mitochondrion may play a role. One is the localization of tritiated acr in the nucleus and in what are probably lipid droplets. The other is the modified behavior of tritiated acr, when extracts of cells grown with acr and hemin are analyzed by paper chromatography. The percentage of modified component is decreased if the cells are grown either without hemin or with the acr-sensitizing factor. There is spectroscopic evidence for an interaction between hemin and acr in solution (Simpson, 1968). While complex formation may partially explain the decreased uptake of acr in the presence of hemin, it does not speak to the problem of changes in the intracellular distribution and form of dye seen in the absence of hemin or in the presence of red cell extract.

The acr-sensitizing factor, now purified free of hemoglobin, removes the resistance of the A strain. The increased sensitivity is concomitant with an increased accumulation of acr in the nucleus of both strains, as shown by electron microscope radioautography as well as by fluorescence microscopy. At the same time there is a change in relative distribution of tritium from cellular extracts analyzed by paper chromatography. Whether this factor is the same as a growth-promoting factor (for *Trypanosoma conorhini*) from red cells, also shown not to be hemoglobin (Deane and Kirchner, 1963), cannot be determined until one or the other has been obtained in reasonably pure state. The chemical nature of a factor negating acquired drug resistance and its mode of action may be of practical as well as biological interest.

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BIBLIOGRAPHY

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

BACHMANN, L., M. M. SALPETER, and E. E. SALPETER. 1968. Das Auflosungsvermogen Elektronen-Mikroskopischer Autoradiographien. *Histochemie.* **15**:234.

BERNHARD, S. A., B. F. LEE, and Z. H. TASHJIAN. 1966. On the interaction of the active site of α-chymotrypsin with chromophores: proflavin binding and enzyme conformation. *J. Mol. Biol.* **18**: 405.

BISHOP, D. G., and R. M. SMILLIE. 1970. The effect of chloramphenicol and cycloheximide on lipid synthesis during chloroplast development in *Euglena gracilis*. *Arch. Biochem. Biophys.* **137**:179.

BOSE, S., B. P. GOLHOSKAR, and K. J. RANADIVE. 1966. Difference in the action of acriflavin and hydroxyurea on synthesis of macromolecules in a mammalian system *in vitro*. *Experientia (Basel).* **22**:509.

BRAUN, W. 1965. Bacterial Genetics. W. B. Saunders Company, Philadelphia. 174–175.

BRAY, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.* **1**:279.

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

DEANE, M. P., and E. KIRCHNER. 1963. Life cycle of *Trypanosoma conorhini*. Influence of temperature and other factors on growth and morphogenesis. *J. Protozool.* **10**:391.

DOZY, A. M., E. F. KLEIHAUER, and T. H. J. HUISMAN. 1968. Studies on the heterogeneity of hemoglobin. XIII. Chromatography of various human and animal hemoglobin types on DEAE-Sephadex. *J. Chromatogr.* **32**:723.

ELORZA, M. V., and R. SENTENDREU. 1969. Effect of cycloheximide on yeast cell wall synthesis. *Biochem. Biophys. Res. Commun.* **36**:741.

FUKUHARA, H., and C. KUJAWA. 1970. Selective inhibition of the *in vivo* transcription of mitochondrial DNA by ethidium bromide and acriflavin. *Biochem. Biophys. Res. Commun.* **41**:1002.

GAUGHAN, P. L., and S. M. KRASSNER. 1971. Hemin deprivation in culture stages of the haemoflagellate, *Leishmania tarentolae*. *Comp. Biochem. Physiol.* **39** (Pt. 1 B):19.

GLAZER, A. N. 1965. Spectral studies of the interaction of α-chymotrypsin and trypsin with proflavin. *Proc. Nat. Acad. Sci. U. S. A.* **54**:171.

GUTTMAN, H., and R. N. EISENMAN. 1965. Acriflavin-induced loss of kinetoplast deoxyribonucleic acid in *Crithidia fasciculata*. *Nature* (*London*). **207**:1280.

HENDERSON, P. J., and H. A. LARDY. 1970. Bongkrekic acid—an inhibitor of the adenine nucleotide translocase of mitochondria. *J. Biol. Chem.* **245**: 1319.

HILL, G. C., and W. A. ANDERSON. 1969. Effects of acriflavin on the mitochondria and kinetoplast of *Crithidia fasciculata*. *J. Cell Biol.* **41**:547.

HILL, G., and W. ANDERSON. 1970. Electron transport systems and mitochondrial DNA in *Trypanosomatidae*: a review. *Exp. Parasitol.* **28**:356.

HUISMAN, T. H. J., and A. M. DOZY. 1965. Studies on the heterogeneity of hemoglobin. IX. The use of Tris (hydroxymethyl) aminomethane HCl buffers in the anion exchange chromatography of hemoglobins. *J. Chromatogr.* **19**:160.

HUISMAN, T. H. J., H. R. ADAMS, M. O. DIMMOCK, W. E. EDWARDS, and J. B. WILSON. 1967. The structure of goat hemoglobin. I. Structural studies of the β chains of the hemoglobins of normal and anemic goats. *J. Biol. Chem.* **242**:2534.

KONSTANTINOVA-SCHLEZINGER, M. A. 1965. Fluorimetric Analysis. N. Kaner, translator. Daniel Davey and Co., Inc., Hartford, Conn.

LAMB, A., G. CLARK-WALKER, and A. LINNANE. 1968. The biogenesis of mitochondria. 4. The differentiation of mitochondrial and cytoplasmic protein synthesizing systems *in vitro* by antibiotics. *Biochim. Biophys. Acta.* **161**:415.

LERMAN, L. S. 1961. Structural considerations in the interaction of DNA and acridines. *J. Mol. Biol.* **3**:18.

LERMAN, L. S. 1963. The structure of the DNA-acridine complex. *Proc. Nat. Acad. Sci. U. S. A.* **49**: 94.

LIZARDI, P. 1971. Studies on the biogenesis of mitochondrial ribosomes from *Neurospora crassa*. Doctoral dissertation. The Rockefeller University, New York.

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

MCILWAIN, H. 1941. A nutritional investigation of the antibacterial action of acriflavine. *Biochem. J.* **35**:1311.

MEYER, R. R., and M. V. SIMPSON. 1969. Differential inhibition of mitochondrial and nuclear DNA polymerases by the mutagenic dyes ethidium bromide and acriflavin. *Biochem. Biophys. Res. Commun.* **34**:238.

MÜHLPFORDT, H. 1964. Über den Kinetoplasten der Flagellaten. *Z. Tropenmed. Parasitol.* **15**:289.

NAGAO, M., and T. SUGIMURA. 1965. Selective inhibition by acriflavin of cytochrome a and b synthesis in yeast undergoing aerobic adaptation. *Biochim. Biophys. Acta.* **103**:353.

NEWTON, B. A. 1957. The mode of action of phenanthridines: the effect of eithidium bromide on cell division and nucleic acid synthesis. *J. Gen. Microbiol.* **17**:718.

NEWTON, B. A. 1964. Mechanisms of action of phenanthridine and amino quinaldine trypanosides. *Advan. Chemother.* **1**:35.

NICHOLSON, B. H., and A. R. PEACOCKE. 1966. The inhibition of ribonucleic acid polymerase by acridines. *Biochem. J.* **100**:50.

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

RENGER, H. C., and WOLSTENHOLME, D. R. 1971. Kinetoplast and other satellite DNAs of kinetoplastic and dyskinetoplastic strains of *Trypanosoma*. *J. Cell Biol.* **50**:533.

RIOU, G. 1970. Specific inhibition by ethidium bromide of the incorporation of ^{3}H-thymidine into the kinetroplast DNA of *Trypanosoma cruzi*. *Biochem. Pharmacol.* **19**:1524.

SALPETER, M. M. 1966. General area of autoradiography at the electron microscopel evel. *In* Methods in Cell Physiology. D. Prescott, editor. Academic Press Inc., New York. **2**:229

SALPETER, M. M., and L. BACHMANN. 1964. Autoradiography with the electron microscope. *J. Cell Biol.* **22**:469.

SALPETER, M. M., L. BACHMANN, and E. E. SALPETER. 1969. Resolution in electron microscope radioautography. *J. Cell Biol.* **41**:1.

SAUNDERS, A. M. 1964. Histochemical identification of acid mucopolysaccharides with acridine orange. *J. Histochem. Cytochem.* **12**:164.

SILVER, S. 1967. Acridine sensitivity of bacteriophage T_2 : a virus gene affecting cell permeability. *J. Mol. Biol.* **29**:191.

SILVER, S., E. LEVINE, and P. M. SPIELMAN. 1968.

Acridine binding by *Escherichia coli*: pH dependency and strain differences. *J. Bacteriol.* **95**:333.

SIMPSON, L. 1968. Effect of acriflavin on the kinetoplast of *Leishmania tarentolae*. *J. Cell Biol.* **37**:660.

STEINERT, M. 1969. Specific loss of kinetoplast DNA in trypanosomatidae treated with ethidium bromide. *Exp. Cell Res.* **55**:248.

STEINERT, M., and S. VAN ASSEL. 1967. The loss of kinetoplastic DNA in two species of trypanosomitidae treated with acriflavin. *J. Cell Biol.* **34**:489.

STEINERT, M., S. VAN ASSEL, and S. STEINERT. 1969. Etude, par autoradiography, des effets du bromure d'ethidium sur la synthèse des acides nucleique de *Crithidia lucilliae*. *Exp. Cell Res.* **56**:69.

STRAUSS, P. R., 1971 *a*. The effect of homologous rabbit antiserum on the growth of *Leishmania tarentolae*—a fine structure study. *J. Protozool.* **18**: 147.

STRAUSS, P. R. 1971 *b*. A strain of *Leishmania tarentolae* resistant to purified acriflavin. *Nature (London).* **230**:49.

STUART, K. 1971. Evidence for the retention of kinetoplast DNA in an acriflavin-induced dyskinetoplastic strain of *Trypanosoma brucei* which replicates the altered central element of the kinetoplast. *J. Cell Biol.* **49**:189.

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.

UDENFRIEND, S. 1962. Fluorescence Assay in Biology and Medicine. Academic Press Inc., New York.

UDENFRIEND, S. 1969. Fluorescence Assay in Biology and Medicine, II. Academic Press Inc., New York.

WEIBEL, E., G. KISTLER, and W. SCHERLE. 1966. Practical stereological methods for morphometric cytology. *J. Cell Biol.* **30**:23.

WEINSTEIN, I. B., and I. H. FINKELSTEIN. 1967. Proflavine inhibition of protein synthesis. *J. Biol. Chem.* **242**:3757.

WERENNE, J., H. GROSJEAN, and H. CHANTRENNE. 1966. Effect of proflavine on the binding of isoleucine to t-RNA. *Biochim. Biophys. Acta.* **129**:585.

WILKIE, D. 1970. Selective inhibition of mitochondrial synthesis in *Saccharomyces cerevisiae* by canavanine. *J. Mol. Biol.* **47**:107.

YOTSUYANAGI, Y. 1962 *a*. Études sur le chondriome de la levure. I. Variation de l'ultrastructure du chondriome au cours du cycle de la croissance aérobie. *J. Ultrastruct. Res.* **7**:121.

YOTSUYANAGI, Y. 1962 *b*. Étude sur le chondriome de la levure. II. Chondriomes des mutants à déficience respiratoire. *J. Ultrastruct. Res.* **7**:141.