ACRIFLAVIN RESISTANCE IN THE HEMOFLAGELLATE, *LEISHMANIA TARENTOLA E*

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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-3H by cells. Metabolism was studied by paper chromatography of aqueous extracts from cells grown with acr-3H, 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 kinetoplast is usually increased, and the cell retains its kinetoplast.
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, 1971b). 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-3H to better assess the localization of the dye in the cell. It also provides further information on the acr-sensitizing factor.

METHODS

Culture

Promastigotes2 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 acriflavine (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 acriflavine (acr) (Strauss, 1971b). 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. Resis-

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).
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 NH₄Cl, 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 (Rf) described by Albert. Instead, the Rf for acr in our hands was 0.63 and the Rf for proflavin was 0.67. The Rf's for acr and proflavin in NH₄Cl 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% aqueous NH₄Cl. 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/mmol.

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 NH₄Cl and butanol/acetic acid systems and compared with acr-³H 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, 1971a). Specimens were examined with an RCA-3F electron microscope at 50 kv. For EM radioautography, cells were grown in 500 ng/ml acr-³H, 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 X 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).

**Acridin-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 | Acridin-Sensitizing Factor (470 ng/ml) | RCE A T | Response of |
|----------------------------------|-------------|---------------------------------------|---------|-------------|
| **Growth properties**            |             |                                       |         |             |
| Lag time                         | + or -      | 36 hr                                 | 24 hr   |             |
| Population doubling              | -           | 12 hr                                 | 12 hr   |             |
| time in log phase                | +           | 12 hr                                 | 12 hr; 24 hr* |             |
| +                                | 21.6 hr     | 21.6 hr                               |         |             |
| **Dyskinetoplasia after 5-7 days growth** |             | <10%                                   | <10%    |             |
|                                 | +           | <10%                                   | ≥40%    |             |
|                                 | +           | >10%                                   | ≥40%    |             |
| **Hemio requirement for ≥50% growth** |             | <613 nM                                | <613 nM |             |
|                                 | +           | 31 nM                                  | ≥ 31 nM |             |
|                                 | +           | 31 nM                                  | > 31 nM |             |
| **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                       |              |             |
|                                 | +           | death at subculture                   |              |             |
|                                 | +           | excellent growth                       |              |             |
|                                 | +           | death at subculture and D              |              |             |
| **Cycloheximide** 1 mm           |             | death at subculture (no D)             | continuous growth |             |
|                                 | +           | death at subculture                    | death at subculture | same as A |
| 0.25 mm                         | -           | continuous growth                      | death and D  |             |
| **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. 3a and 3b. 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 | kinetoplast only | same as A |
| + Acriflavin, 470 ng/ml | kinetoplast, cytoplasm and nucleus | same as A |
| + Acriflavin, 470 ng/ml + red cell extract (RCE) | | |
| Acriflavin-3H uptake | progressive with growth | same as A |
| −RCE | 2 times amount as without RCE at all times | same as A |
| +RCE | | |
| Acriflavin-3H distribution | mitochondrion and kinetoplast; cell surface; lipid; nucleus | same as A |
| −RCE | mitochondrion and kinetoplast; cell surface; lipid; some movement into nucleus | same as A |
| +RCE | | |
| Morphological changes during division | not so stubby as T during division process | stubby by 36 hr, elongated again by 72 hr |

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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.
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
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)*

Each figure is the average of two flasks.

| Condition | Time  | (0 N + 0 K)/(2 N + 0 K) | (1 N + 0 K)/(2 N + 0 K) | 2 N + 1 K | 2 N + 2 K | 1 N + 2 K | Total DF | Cells/ml |
|-----------|-------|-------------------------|-------------------------|----------|----------|----------|---------|---------|
| +Hemin, -acr | 0     | 1.62                    | 1.96                    | 5        | 5        | 5        | 12.5    | 1.74    |
|            | 4     | 2                       | 4                       | 2        | 4        | 2        | 10.0    | 1.38    |
|            | 8     | 2                       | 6                       | 1        | 2        | 1        | 10.0    | 1.04    |
|            | 12    | 4                       | 4                       | 2        | 2        | 1        | 9.0     | 1.12    |
|            | 15    | 3                       | 6                       | 2        | 1        | 2        | 10.0    | 1.26    |
|            | 18    | 5                       | 2                       | 6        | 1        | 2        | 9.0     | 1.52    |
|            | 21    | 5                       | 2                       | 5        | 2        | 1        | 8.0     | 1.88    |
|            | 24.3  | 6                       | 3                       | 3        | 2        | 9        | 18.0    | 2.42    |
|            | 27    | 5                       | 5                       | 5        | 3        | 14       | 22.0    | 2.74    |
|            | 30    | 1                       | 2                       | 8        | 1        | 11       | 20.0    | 3.12    |
|            | 33    | 2                       | 3                       | 9        | 4        | 16       | 28.0    | 3.72    |
|           | Average: 3.0 |                           |                         |          |          |          |         |         |
| -Hemin, -acr | 0     | 1.74                    | 1.56                    | 4        | 4        | 4        | 13.0    | 1.92    |
|            | 4     | 9                       | 7                       | 2        | 1        | 1        | 9.0     | 1.66    |
|            | 8     | 8                       | 7                       | 4        | 3        | 1        | 11.0    | 1.52    |
|            | 12    | 7                       | 3                       | 3        | 3        | 1        | 9.0     | 1.32    |
|            | 15    | 10                      | 4                       | 3        | 3        | 2        | 15.0    | 2.12    |
|            | 18    | 5                       | 3                       | 4        | 2        | 9        | 15.0    | 2.30    |
|            | 21    | 9                       | 8                       | 5        | 2        | 14       | 24.0    | 2.94    |
|            | 24.3  | 3                       | 2                       | 3        | 3        | 8        | 15       | 2.42    |
|            | 27    | 6                       | 2                       | 6        | 3        | 14       | 23       | 2.74    |
|            | 30    | 6                       | 4                       | 4        | 6        | 14       | 23       | 3.12    |
|            | 33    | 5                       | 2                       | 2        | 2        | 6        | 13       | 3.72    |
|           | Average: 3.1 |                           |                         |          |          |          |         |         |
| +Hemin, +acr | 0     | 1.56                    | 1.56                    | 6        | 6        | 6        | 18.0    | 1.74    |
|            | 4     | 7                       | 7                       | 2        | 2        | 2        | 13.0    | 1.46    |
|            | 8     | 7                       | 7                       | 4        | 2        | 9        | 16.0    | 1.88    |
|            | 12    | 5                       | 6                       | 1        | 2        | 2        | 9.0     | 1.92    |
|            | 15    | 11                      | 11                      | 4        | 2        | 8        | 17.0    | 1.66    |
|            | 18    | 7                       | 7                       | 4        | 1        | 3        | 11.0    | 1.52    |
|            | 21    | 11                      | 12                      | 2        | 5        | 1        | 18.0    | 2.30    |
|            | 24.3  | 12                      | 12                      | 2        | 2        | 1        | 7.0     | 2.48    |
|            | 27    | 18                      | 18                      | 5        | 6        | 3        | 18.0    | 2.66    |
|            | 30    | 10                      | 10                      | 6        | 6        | 2        | 18.0    | 3.04    |
|            | 33    | 18                      | 18                      | 4        | 3        | 1        | 13       | 3.26    |
|           | Average: 3.8 |                           |                         |          |          |          |         |         |
| -Hemin, +acr | 0     | 1.68                    | 1.68                    | 8        | 8        | 8        | 15.0    | 1.52    |
|            | 4     | 8                       | 9                       | 3        | 2        | 3        | 15.0    | 1.52    |
|            | 8     | 9                       | 9                       | 2        | 1        | 2        | 11.0    | 1.40    |
|            | 12    | 12                      | 12                      | 3        | 2        | 7        | 11.0    | 1.14    |
|            | 15    | 5                       | 5                       | 3        | 6        | 5        | 16.0    | 1.08    |
|            | 18    | 12                      | 12                      | 3        | 1        | 2        | 8.0     | 1.20    |
|            | 21    | 13                      | 13                      | 3        | 2        | 1        | 8.0     | 1.36    |
|            | 24.3  | 13                      | 13                      | 3        | 1        | 2        | 8.0     | 1.36    |
|            | 27    | 16                      | 16                      | 2        | 3        | 4        | 9.0     | 1.24    |
|            | 30    | 16                      | 16                      | 5        | 3        | 1        | 9.0     | 1.82    |
|            | 33    | 20                      | 20                      | 6        | 3        | 4        | 12.0    | 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](image-url)  
**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 (1971a). 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, 1962a, 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 and finally remained only as a smudge (Figs. 11–12).

**Acriflavine 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, 1971b) 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% NH₄Cl 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 *Rf* of 0.63, the same as the *Rf* of the tritiated dye and of the purified acr standard. The second peak had an *Rf* of less than 0.05. The minor component had an *Rf* 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, 1971b). In order to substantiate the fluorescence observations, it was decided to perform electron microscope radio-
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. X 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. X 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. X 18,000.
TABLE IV

| Strain | Hemin concentration µg/ml | cpm/10⁶ cells |
|--------|---------------------------|---------------|
| 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-3H 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.

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 | 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 | Nucleus a = (2)/(1) | Cytoplasm b = (3)/(1) | Surface c = (4)/(1) | Mitochondrion d = (5)/(1) | Kinetoplast e = (6)/(1) | Flagellar pocket f = (7)/(1) | Lipid droplets g = (8)/(1) | Cytoplasm only h = (9)/(1) |
|------------------------|---------------------|-----------------------|---------------------|--------------------------|------------------------|---------------------------|--------------------------|---------------------------|
| 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.

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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 | 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     |
| 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     |
| T+acr+RCE, 2 days      | 9.0/9.7                  | 7.6/6.5         | 0.5/0.42    | 4.9/2.5          | 1.5/0.28       | 23.2/77.30     |
| A+acr+RCE, 2 days      | 9.4/8.6                  | 9.1/6.7         | 0.4/0.19    | 4.4/2.6          | 1.3/0.47       | 22.1/80.74     |
| 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     |
| A+acr, 5 days          | 13.9/13.5                | 27.2/6.5        | 0.6/0.34    | 4.0/2.2          | 21.2/3.3       | 9.8/79.46      |

* 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 | Whole cell (1) | Nucleus (2) | Total cytoplasm (3) | Mitochondrion (5) | 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         | 1,252       | 11,825              | 974               | 56              | 394                  | 45                |
| T+acr+RCE, 2 days      | 14,925         | 1,452       | 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         | 1,552       | 12,350              | 1026              | 59              | 383                  | 268               |
| A+acr, 5 days          | 12,100         | 1,038       | 10,875              | 810               | 51              | 333                  | 312               |
| T+acr+RCE, 5 days      | 16,475         | 2,217       | 14,760              | 1,070             | 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. (c) 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.)
**Crude Grain Count Data: Grains Associated within the 70% Probability Radius with the Given Category**

| Experimental condition | Total grains counted | Total grain cell associated | Grains associated with nuclei | Grains associated with cytoplasm | Surface grains | Mitochondrial grains | Kinetoplast grains | Flagellar pocket grains | Lipid droplet grains | Cytoplasm only grains |
|------------------------|----------------------|-----------------------------|------------------------------|---------------------------------|----------------|---------------------|---------------------|------------------------|----------------------|----------------------|
| 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      | 2135                 | 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, 1971b; 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-3H, it was suggested that the substance affecting the response to acr was not hemoglobin but some minor component of red cells (Strauss, 1971b).

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. X 6900. (b) T + 500 ng/ml acr, 5 days. X 10,100. (c) T + 500 ng/ml acr, 5 days. X 8700. (d) T + 500 ng/ml acr + RCE, 5 days. X 9600. (e) T + 500 ng/ml acr + RCE, 5 days. X 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.

Phyllis R. Strauss  Acriflavine Resistance
globin peak (Fig. 16). The trailing peak, which comprised less than 0.17% 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.

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 X 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.

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**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.

**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.
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, 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 + a₃ 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 (Gutman 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-

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3 Bongkrekic acid was kindly supplied by Professor W. Berends.
increased accumulation of acr in the nucleus of
The increased sensitivity is concomitant with an
hemoglobin, removes the resistance of the A strain.
extract.
intracellular distribution and form of dye seen in
posed. At least two observations suggest that com-
et al.,
dividing populations such as those obtained in the
for significant dyskinetoplasia in apparently non-
spora crassa
does not speak to the problem of changes in the
decreased uptake of acr in the presence of hemin, it
complex formation may partially explain the
hemin and acr in solution (Simpson,
spectroscopic evidence for an interaction between
hemin or with the acr-sensitizing factor. There is
components in addition to kinetoplast-mitochondrion
acriflavin is more complex than previously sup-
In any case it seems clear that the action of
acriflavin is more complex than previously sup-
posed. At least two observations suggest that com-
ponents 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
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 prac-
tical as well as biological interest.
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