EFFECT OF BERENIL ON NUCLEIC ACID SYNTHESIS IN TRYPANOSOMA BRUCEI

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Abstract

We have examined the activity of diminazene (Berenil) on growth and nucleic acid synthesis in bloodstream forms of Trypanosoma brucei. Our findings indicate that exposure to drug in vivo results in the accumulation of what appear to be predivisional forms which contain ∼2X the amounts of DNA, RNA and protein found in non-exposed parasites. Diminazene [diminaz.] reversibly inhibits the incorporation of $^{3}$H-thymidine and $^{3}$H-uracil into DNA and RNA respectively when trypanosomes are labeled in vivo or in vitro. Diminazene inhibits the activity of DNA polymerases (Pol. 1) but does not appear to inhibit an E. coli RNA polymerase. We conclude that diminazene blocks cell division in T. brucei, inhibits DNA synthesis in these forms and interferes with RNA metabolism by inhibiting synthesis and/or enhancing degradation.

The present study was undertaken to examine (a) the effect of diminazene on the growth and cellular morphology of monomorphic bloodstream trypanosomes; (b) the effect of diminazene on nucleic acid and protein synthesis of these trypanosomes; and (c) the effect of diminazene on prokaryotic and eukaryotic in vitro nucleic acid synthesizing systems. The results obtained are considered as to whether they may account for the ability of this drug to cure some of the African trypanosomiases.
Diminazene has been cited as being kinetoplast selective (Newton, 1967; Newton and LePage, 1968; MacAdam and Williamson, 1969, 1972; Brack et al., 1972a, 1972b; Newton, 1975). Previous investigations have not examined the mode of action of diminazene on African bloodstream trypanosomes. Though its apparent selectivity for (KP-DNA) kinetoplast-DNA is an interesting drug property, this may not be a principle feature responsible for its mode of action in vivo, especially in view of the fact that the kinetoplast-mitochondrion complex of brucei subgroup forms in blood appear to be inactive and that drug-induced dyskinetoplastic bloodstream trypanosomes are viable (Stuart, 1971).

The interaction of diminazene with nucleic acids in vitro suggests that inhibition of nucleic acid and/or protein synthesis may occur in vivo. The present study was undertaken to determine whether diminazene inhibits nucleic acid synthesis in monomorphic trypanosomes multiplying in the mouse bloodstream, or maintained in vitro in a glucose buffer. We have also examined the action of diminazene on prokaryotic and eukaryotic in vitro DNA synthesizing systems. The results obtained are considered as to whether the effect(s) of diminazene on nucleic acid synthesis in bloodstream forms may account for its curative properties on some of the African trypanosomiases.

MATERIALS AND METHODS

Radiochemicals: Thymidine-6-^{3}H(Tdr) (sp. act. 9.82 Ci/mM), Uracil-6-^{3}H(U) (sp. act. 27.5 Ci/mM) and uniformly labeled L-leucine-^{14}C (^{14}C-leu) sp. act. 255 mCi/mM) were purchased from New England Nuclear,
Boston, Mass. Uracil-2,6-\(^{14}\)C \((^{14}\)C-U\) (sp. act. 115 mCi/mM) was purchased from Mallinkrodt Chemicals, St. Louis, Mo. [Methyl-\(^{3}\)H]dTTP \((^{3}\)H-dTTP\) (sp. act. 75 Ci/mM) and \(^{3}\)H-5-UTP \((^{3}\)H-UTP\) (sp. act. 4 Ci/mM) were purchased from International Chemical and Nuclear Corp., Irvine, Calif. Biochemicals: E. coli B DNA polymerase (DNA deoxynucleotidyl transferase, EC 2.7.7.7) Fraction VII, sp. act. 5000 U/mg and calf thymus DNA polymerase EC 2.7.7.7, sp. act. 100 U/mg were purchased from General Biochemicals, Chagrin Falls, Ohio. M. lysodeikticus DNA polymerase, EC 2.7.7.7 sp. act. 100 U/mg was purchased from Miles Laboratories, Kankakee, Ill. All other biochemicals, including E. coli K-12 RNA polymerase, EC 2.7.7.6, sp. act. 600 U/mg, highly polymerized calf thymus DNA, yeast sRNA, pyruvate kinase, deoxyribonuclease I (EC 3.1.4.5, sp. act. 2000 U/mg, and all biochemicals used in polymerase assays were purchased from Sigma Chemical Co., St. Louis, Mo. All other chemicals used were reagent grade and purchased from commercial sources.

Diminazene (4,4'-diamidino-diazo-amino-benzene diaceturate) was a generous gift of Dr. A. H. Loewe, Farbewerke-Hoechst, Frankfurt-am-Main, Germany; it was used without further purification. Source of Trypanosomes and Mice: The strain of Trypanosoma brucei used in these investigations (monomorphic, rodent-adapted) was a generous gift of Dr. W. Trager, Rockefeller University, New York. The infection was maintained in CFI (males, 25-30 gm, 6-8 weeks old - Carworth Farms, N.Y., N.Y.) and NLW mice (males, 25-30 gm, 6-8 weeks old - National Laboratory Animal Co., St. Louis, Mo.) Buffers: (all values as g/l): Buffer 1: EDTA 5.0; Trizma base 5.0; dextrose 2.0; NaCl 4.0; KCl 0.2; adjusted to pH 7.5
with HCl. Buffer 2: Trizma base 5.0; dextrose 2.0; NaCl 4.0; KCl 0.2; CaCl$_2$ 0.2; MgCl$_2$ 6H$_2$O 0.2, adjusted to pH 7.5 with HCl. Harvest of *Trypanosomes*: *Trypanosoma brucei* was maintained in CF1 and NLW mice by intraperitoneal (ip) syringe passage of infected mouse blood. Infection and diminazene cure proceed similarly in both strains of mice. Experimental animals were infected ip with an appropriate number of trypanosomes so as to produce a parasitemia of 1-2 $10^8$ trypanosomes/ml blood within 36 hr. No experimental animals were used longer than 48 hours postinfection. The generation time of this stabulate in the mouse strains used is 5-5.5 hours. Parasitemia levels were routinely determined by hemocytometer count and by examination of wet mounts.

Purification of trypanosomes from cardiac blood was accomplished as described by Lanham (1968). Preparation for Transmission Electron Microscopy: Trypanosome pellets (either before or after DEAE-cellulose filtration) were fixed in cold 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer the pellets were post-fixed with 1% Os$_4$ in the same buffer for 1 hr. All subsequent steps were carried out at room temperature. The pellets were fragmented into small pieces and overlayersed with 1% aqueous uranyl acetate for 20 min, after which the uranyl acetate was withdrawn and the material overlayersed with 70% ethyl alcohol. The pellet fragments were dehydrated by passage through a graded series of alcohols followed by immersion in propylene oxide. The material was embedded in epon (Luft, 1961) which was polymerized in a 60°C oven for 48 hr.

After polymerization, thick sectioning (0.5μ) and staining (0.2% Azure II in 1% sodium borate), sections were examined under a light
microscope for gross evaluation of cell preservation and for the selection of suitable areas for thin sectioning. Thin sections were cut with glass or diamond knives on an MT-2 Porter-Blum ultramicrotome and transferred to formvar-coated grids previously stabilized with a thin film of carbon. Sections were stained with 3% uranyl acetate in 50% ethyl alcohol for 10 min followed by 0.4% lead citrate in 0.1M NaOH for 5 min. Electron micrographs were taken with a Jelco TM or a Phillips 300 electron microscope. **Diminazene Treatment and Labeling:** Diminazene was dissolved in buffer 2 and administered in a small volume (0.2 ml) to mice by the ip route. Thymidine-6-³H or uracil-6-³H (sterile, aqueous) was administered ip (0.2–0.5 mCi/mouse in 0.1–0.3 ml). DEAE purified trypanosomes were suspended in buffer 2 at a concentration of 10⁷ cells/ml. Twenty-five ml aliquots of cells were placed in 50 ml flasks, warmed to the appropriate temperature and diminazene (100 µl of an appropriate concentration dissolved in buffer 2) was added. Two min later, ³H-TdR, ³H-U or ¹⁴C-leu was added to the flasks. Pulse and chase experiments were performed by exposing the trypanosomes for an appropriate length of time to ³H-U and then adding a 1000 fold excess of unlabeled uracil to the suspending medium.

Serum isotope levels in diminazene treated and untreated infected mice were determined as follows: Twenty µl samples of tail blood collected in heparinized capillary tubes at various times after isotope injection were centrifuged in a hematocrit centrifuge and 5 µl samples of sera spotted on Millipore filters. After drying the filters were counted in a Nuclear Chicago Mark II liquid scintillation counter using a toluene based scintillation fluid.
Radioactivity Measurements of Trypanosomes: (a) Whole cells: $5 \times 10^6$ or $10^7$ DEAE-purified trypanosomes in buffer 2 were poured onto a Millipore filter (25 mm., 0.45μ pore size) and washed three times with buffer 2. (b) Nucleic acids: $5 \times 10^6$ or $10^7$ DEAE purified trypanosomes in buffer 2 were diluted with an equal volume of cold 10% TCA and placed on ice for a minimum of 15 minutes. The TCA precipitates were collected on Millipore filters and washed with four volumes of cold 5% TCA (Munro and Fleck, 1966).

The relative amount of label appearing in DNA and RNA was determined by comparing counts in TCA precipitates ((b) above) with counts remaining in TCA precipitates after base hydrolysis as follows: TCA precipitates as obtained in (b) above were centrifuged at 1465×g for 15 minutes. The precipitates were washed with 5% TCA and resuspended in 2.5 ml of 0.5N NaOH. After a 2 hour incubation at 37°C to hydrolyze RNA, the solutions were chilled, and 250 μg of BSA (in 1 ml H₂O) was added. After neutralization with 2.5 ml of 0.5N HCl, 6 ml of cold 10% TCA were added. The TCA precipitates were collected on Millipore filters and washed several times with 5% TCA. (c) Protein: TCA precipitates obtained in (b) above were boiled for 20 minutes before Millipore filtration (Munro and Fleck, 1966).

All Millipore filters were placed in glass vials, dried, and counted by the scintillation method. Chromatography of Nucleic Acid bases: Trypanosomes labeled in vivo or in vitro with thymidine-6-$^3$H, uracil-6-$^3$H or uracil-2,6-$^{14}$C were isolated and purified as previously described. After passage through DEAE cellulose the cells were centrifuged at 1020×g for 10 minutes and the pellets resuspended in a final volume of 1.2 ml of buffer 2 to which 2 ml of 7% HClO were added. The solution was placed on
ice for 3 min and the ppt's centrifuged at 1465xg for 10 min. The HClO₄ ppt's were washed 2X with a 2% HClO₄ solution containing 2.0x10⁻³M sodium pyrophosphate. The washed ppt. was hydrolyzed in 70% HClO₄ for 1 hr at 100°C. The hydrolysate was chilled, neutralized with KOH and frozen. The hydrolysate was removed from the freezer and centrifuged at 1465xg for 10 minutes to remove precipitated KClO₄. The clear supernatant was mixed in various proportions with a solution containing 0.4 mg/ml each of adenine, thymine, cytosine, uracil and guanine. The mixture of the HClO₄ hydrolysate and bases (3μl) was spotted on Eastman cellulose thin layer plates (2 dimensional chromatograms) or Whatman #1 filter paper (one dimensional chromatograms) and chromatographed in one or two dimensions using the following solvent systems: Solvent (1) Propan-2-ol (680 ml), 11.6 N HCl (176 ml), water to 1 liter; Solvent (2) butan-1-ol (770 ml) water (130 ml), 98% formic acid (100 ml) (Littlefield and Dunn, 1958). Spots on the two dimensional chromatograms corresponding to the five bases were located using a short-wavelength U.V. lamp, scraped off, placed in glass vials and counted by the liquid scintillation method.

After development in Solvent 1, the one-dimensional chromatograms were dried and the spots corresponding to the five bases were located by UV. The paper was cut into 0.5" strips, and counted by the scintillation method. The location of radioactivity was compared with the location of the spots. Measurement of DNA, RNA and Protein: DNA was determined by the P-nitrophenylhydrazine (PNPH) method of Webb and Levy (1955). RNA was determined by the orcinol method (Schneider, 1957). DNA and RNA content were determined on 5% TCA hydrolysates (30 min 100°C) of purified trypanosomes. Protein was determined by the method of Lowry (1951) using
homogenates of purified trypanosomes. **DNA Polymerase Assay:** DNA polymerase was assayed by the filter paper disk technique of Bollum (1966) as described by Brown and Coffey (1972). **RNA Polymerase Assay:** RNA polymerase was assayed by the method of Chamberlain and Berg (1962) modified as follows. The standard assay system contained the following constituents in a final volume of 0.1 ml: 4 μmoles Tris-HCl, pH 7.9 at 37°C; 0.4 μmoles MgCl₂; 0.1 μmole MnCl₂; 1.2 μmoles β-mercaptoethanol; 0.04 μmole each of ATP, CTP and GTP; 0.04 μmole UTP containing 0.5 μCi of 3H-UTP; 0.5-2.0 units of RNA polymerase and 1-10 μg of native or heat denatured calf thymus DNA. The mixture was incubated at 37°C for 30 minutes. The reaction was stopped by placing the reaction mixture on a filter paper disk at 50°C (Bollum, 1966). The paper disk was dried and washed as in the DNA polymerase assay except that in the second washed 0.01% ATP was replaced by 0.01% UTP. After drying the radioactivity on the disks was determined by the scintillation method.
RESULTS

Effect of Diminazene on the Growth of Trypanosoma brucei in vivo.

When a minimum curative dose (MCD) is administered to mice showing \(10^8\) organisms/ml blood this parasitemia level persists for 12-18 hr. The trypanosomes are motile but larger than those from untreated animals. The parasitemia level decreases after 18 hr and full clearing is achieved within one to four days after treatment. The rate of clearing appears to be directly dependent on the amount of diminazene administered, in the range 10-100 \(\mu\)g/g. The MCD in these studies was \(5\) \(\mu\)g/g body weight. The smallest dose that resulted in >90% cure when administered two generation times before death would have occurred was 10 \(\mu\)g/g, defined as the curative dose (CD).

Ultrastructural Alterations in Diminazene Treated T. brucei In Vivo:

Examination of T. brucei harvested at different times after treatment with a CD reveals a sequence of changes. Alterations in the KP-DNA are seen soon after treatment, whereas nucleolar changes are observed only after the elapse of several generation times. After four hours the KP-DNA in nearly all cells seems to have lost its normal filamentous rod-line structure (Figs. 3 and 4) and appears fragmented (Figs. 5a and 5b). At this time, the cell nucleus and nucleolus are indistinguishable from those seen in untreated cells (Figs. 1, 2 and 6). Trypanosomes examined after 18 hr or \(\sim 3.5\) generation times show pronounced alterations in the nucleus. The nucleolus appears either as one or more highly condensed spheres (in about 80% of the cells) or
as a number of small fragments (in about 20% of the cells). The KP-DNA is fragmented in all cells (Figs. 7a, 7b and 7c).

MacAdam and Williamson (1972) found alterations in the KP-DNA of *T. rhodesiense* 6 hr after diminaz treatment. No alterations in the nucleus or nucleolus were evident at this time.

DEAE purified trypanosomes resuspended in buffer 2 containing diminazene (2-10 µg/ml) survive for 3-4 hr at 25°C. After a 2 hr exposure to 10 µg/ml nearly all cells exhibit fragmented KP-DNA. The nucleus and nucleolus are unaltered. In vitro exposure of trypanosomes to 30-100 µg/ml results in rapid cell death followed by lysis.

It has been reported that nucleolar fragmentation immediately precedes cell division in *T. brucei* (Vickerman and Preston, 1970). Nucleolar fragmentation was rarely observed during electron microscopic examination of untreated trypanosomes. The large number of predivisional forms seen after 18 hr suggested that diminaz treated cells were unable to divide. Since a blockage in cell division may be caused by a breakdown of essential steps during the S and M phases of the cell cycle, we next examined nucleic acid synthesis in vivo. **Effect of Diminaz on Incorporation of **$^3$H-Thymidine and $^3$H-Uracil into DNA and RNA In Vivo:** Availability of $^3$H-TdR $^3$H-U to bloodstream trypanosomes in untreated and diminaz treated animals. Figures 8 and 9 show the levels of $^3$H-TdR and $^3$H-U respectively in the sera of untreated and diminaz treated mice at various times after ip. injection of isotope. Diminaz does not interfere with the uptake of $^3$H-TdR or $^3$H-U from the peritoneum nor does its presence alter the rate at which
radioactivity disappears from the bloodstream. This observation holds
when injection of $^{3}$H-TdR follows diminaz administration by no less
than 30 min., and $^{3}$H-U follows diminaz by no less than 60 min. Effect
of diminaz on $^{3}$H-TdR incorporation. Figure 10 shows the effect of a
cd on incorporation of $^{3}$H-TdR into cold 5% TCA precipitable, alkali
stable material (DNA) at various times after injection of $^{3}$H-TdR. There
was no loss of radioactivity in TCA precipitates from trypanosomes
labeled in vivo with $^{3}$H-TdR following alkaline hydrolysis. The only
radioactive material recoverable from 70% PCA hydrolysates of dilute
acid insoluble extracts of trypanosomes labeled with $^{3}$H-TdR was $^{3}$H-T.
(see Fig. 22). After a 30 min exposure to drug, incorporation of
$^{3}$H-TdR into trypanosome DNA is inhibited by 70-80%. The greatest in-
corporation takes place shortly after injection of the $^{3}$H-TdR, and the
rate of incorporation decreases as a function of time. This labeling
pattern is attributable to the continuously decreasing level of isotope
in the serum. During the time course of the experiment described in
Figure 10, (0.5-3.0 hours after $^{3}$H-TdR injection) trypanosomes in un-
treated mice incorporated 8-9X more $^{3}$H-TdR than did trypanosomes in
diminaz treated mice. When one-tenth the amount of diminaz (1 μg/g
body weight) is administered incorporation of $^{3}$H-TdR into trypanosome
DNA is 70-75% of the control level.

As with other diamidines, the serum level of diminaz quickly
reaches a maximum and falls rapidly after ip injection (Hawking, 1963).
The experiment described in Figure 11 was performed to determine
whether diminaz. inhibition of $^3$H-TdR incorporation was reversible. When $^3$H-TdR is given 4-5 hr after a [cd], inhibition of incorporation is substantially (80% vs 40%) less than the inhibition observed 30 min after diminaz. treatment. These results indicate some resumption of $^3$H-TdR incorporation several hours after diminaz. treatment and suggest that diminaz. inhibition of $^3$H-TdR incorporation in vivo is reversible. Effect of diminaz. on $^3$H-U incorporation. Figure 12 shows the effect of a cd on the incorporation of $^3$H-U into cold 5% TCA precipitable-alkali unstable material (RNA) at various times after exposure of trypanosomes to $^3$H-U in vivo. Alkaline hydrolysis removed 95% of the radioactivity from TCA precipitates. The radioactive material recoverable from 70% PCA hydrolysates of dilute acid insoluble extracts was almost exclusively uracil and cytosine. Only a small amount of radioactive thymine (<5%) was detected (see Fig. 23). The sum of the synthesis and degradation of RNA occurring in the presence of a continuously decreasing level of $^3$H-U is represented by the curves in Figure 12. Much of the RNA labeled during the pulse is believed to be m-RNA, a conclusion derived from the amount of label disappearing from RNA between 3 and 7 hr after administration of $^3$H-U.

About 60% less $^3$H-U is incorporated into rapidly labeled RNA in trypanosomes in Berenil treated vs. untreated mice (Fig. 12). When 1µg/g diminaz. is given the inhibition of incorporation of $^3$H-U into rapidly labeled RNA is reduced to 20% of the control or is 80% of control level.

The experiment described in Figure 13 was performed to determine whether diminaz. inhibition of $^3$H-U incorporation is reversible.
Effect of Diminaz on the Ratio of DNA/RNA/Protein in T. brucei in vivo.

Table I shows that the DNA, RNA and protein content of bloodstream trypanosomes is doubled after 12 hours of treatment (-2.1-2.2 generation times) with a cd. The values for untreated trypanosomes are similar to those reported by Riou and Pautrizel (1969). These data also suggest that Berenil treated trypanosomes double their DNA, RNA and protein content, but are unable to divide. Effect of Diminaz on Incorporation of 3H-Thymidine, 14C-Uracil and 14C-Leucine into DNA, RNA and Protein In Vitro. DEAE purified trypanosomes suspended in buffer 2 are metabolically active and motile for 3-4 hr at 37°C (at 3.5 hr, 50% of the cells are non-motile) and 5-6 hr at 25°C (at 6 hr, 50% of the cells are non-motile). The incorporation of nucleic acid and protein precursors under these conditions probably reflects a minimal (endogenous) synthesis. Trypanosomes suspended in Dulbecco's medium plus fetal calf serum survived no longer than in buffer 2. These in vitro studies were not performed under sterile conditions. Incorporation of 3H-TdR and 14C-Leu in vitro were unaffected by penicillin (100 U/ml) and streptomycin (100 µg/ml). The amount of label incorporated was directly proportional to the concentration of trypanosomes in the suspending medium indicating no significant bacterial contamination.

Figure 14 shows that the small amount of 3H-TdR incorporation into trypanosome DNA (cold 5% TCA precipitable, alkali stable material) which occurs at 25°C in buffer 2 is inhibited by Berenil. Figure 15 shows that the appreciable incorporation of 3H-TdR into trypanosome DNA which occurs at 37°C in buffer 2, is completely inhibited by 5µg/ml diminaz. Figure 16 suggests that diminaz inhibition of 3H-TdR incorporation in vitro
is reversible, i.e. 30 minutes after cessation of $^3$H-TdR incorporation, cells that are washed and resuspended without diminaz. resume incorporation of $^3$H-TdR. These in vitro results are in agreement with the in vivo findings.

Figure 17 reveals that appreciable incorporation of $^{14}$C-U into trypanosome RNA (cold 5% TCA precipitable, alkali unstable material) occurs at 25°C in vitro. In the presence of diminaz. (10 and 20 μg/ml), inhibition of incorporation in control cells (trypanosomes isolated from untreated mice) is not apparent for at least 30 minutes. Trypanosomes exposed to a curative dose of diminaz. in vivo for 30 minutes, prior to being harvested, do not incorporate label whether or not diminaz. is added to the incubation medium. However, cells re-suspended in the absence of diminaz. (plus diminaz. in vivo but minus diminaz. in vitro) incorporate $^3$H-U into RNA at nearly the same rate as control cells, after a short lag period. Incorporation of $^3$H-U into RNA proceeds without a lag for only a short time at 37°C (see Figure 18) after which no increase in the amount of label in RNA is detected. The incorporation at 37°C is inhibited by diminaz.

Figure 19a-d show the results of a series of pulse and chase experiments with $^{14}$C-uracil. These results suggest that: (i) RNA synthesized in the absence of diminaz. is degraded more quickly in the presence of diminaz. than in the absence of diminaz., i.e. pre-existing RNA is degraded more rapidly in the presence of diminaz. than in the absence of diminaz., (ii) RNA synthesized in the presence of diminaz. is not degraded as rapidly as pre-existing RNA when diminaz. is present,
i.e. RNA synthesized during exposure to diminaz is more stable to degradation than RNA synthesized in the absence of diminaz. Figures 20 and 21 show the effect of diminaz on incorporation of $^{14}$C-leucine into cold 5% TCA precipitable, hot 5% TCA stable material at 25°C and 37°C in vitro. At both temperatures, diminaz appears to stimulate the incorporation of $^{14}$C-Leu into protein.

These in vitro results suggest that Berenil interferes with DNA and RNA synthesis. The effect of Berenil on isolated in vitro DNA and RNA synthesizing systems was examined next.

Chromatography of Trypanosome PCA Hydrolysates: One Dimensional Chromatograms. Figure 22 depicts the radioactive bases derived from trypanosome nucleic acids labeled either in vivo or in vitro with $^{3}$H-thymidine. In both cases, the only radioactive base recovered from 70% PCA hydrolysates was thymine. When trypanosomes were labeled in vivo with $^{3}$H-uracil (Fig. 23) labeled cytosine and uracil (in approximately equal amounts) and a trace ($<$5%) of labeled thymine were recovered from the 70% PCA hydrolysate. When trypanosomes were labeled in vitro at 25°C or 37°C with $^{14}$C-uracil (Figs. 24 and 25) most of the radioactivity recovered from the PCA hydrolysates was present as uracil, a small amount was present as cytosine and about 25% was present as thymine. Two Dimensional Chromatograms. Two dimensional chromatograms were developed (five hours in solvent 1 and six hours in solvent 2 at room temperature) and analyzed for cells labeled in vivo with either $^{3}$H-thymidine on $^{3}$H-uracil. The location of the radioactivity is shown in Table II. The $R_f$ values of the five nucleic acid bases in the two solvent systems are shown in Table III. These data suggest that (i) the metabolic fate of thymidine in vivo and in vitro is the same and (ii) trypanosomes convert much less uracil to cytosine and
more to thymine when they are maintained in vitro. Effect of diminaz on DNA Polymerase: The DNA polymerase catalyzed incorporation of $^3$H-dTTP into DNA was examined using three different DNA polymerases, two bacterial and one mammalian. Omission of template DNA or polymerase from the standard reaction mixture reduced incorporation of $^3$H-dTTP by 98%. Omission of one nucleoside-5'-triphosphate from the reaction mixture reduced the amount of DNA synthesized by 70% and omission of Mg$^{++}$ from the reaction mixture reduced the amount of DNA synthesized by 80%. The Mg$^{++}$ optimum is 8-10 mM. The radioactivity incorporated was sensitive to DNAase. In this system DNA is saturating at ~100 μg/ml for the E. coli enzyme at ~66 μg/ml for the M. lysodeikticus enzyme and at ~88 μg/ml for the calf thymus enzyme. With these polymerases the reaction proceeds at a constant rate for at least 90 min. The reaction was stopped by pipetting the reaction mixture onto filter paper disks, previously warmed to 50°C. The reaction may also be stopped by the addition of diminaz (100 μg/ml) to the reaction mixture. When the time dependence of the reaction was examined using diminaz to stop the reaction at appropriate times, and all reaction mixtures were placed on disks after 90 min, the results were those seen in Figure 26. These data indicate that diminaz stops DNA synthesis instantaneously but does not cause degradation of previously synthesized DNA.

Figure 27 reveals that diminaz inhibits the activity of the three DNA polymerases. The bacterial enzymes are more sensitive to drug (50% inhibition is achieved at 5 μg/ml diminaz) than is the mammalian enzyme (50% inhibition is achieved at 10 μg/ml diminaz). At 5 μg/ml diminaz, the E. coli enzyme was also inhibited by 50% when E. coli heat denatured
DNA replaced heat-denatured CT-DNA as template. The experiments described in Figures 28-30 were performed to determine the diminaz-sensitive reaction constituent(s). The ability of either additional enzyme, additional DNA, or sRNA to relieve diminaz inhibition was examined. Figures 28 and 29 reveal that in the case of the bacterial enzymes, only the addition of enzyme effectively overcomes inhibition. Figure 30 shows that inhibition of the mammalian enzyme is overcome by addition of either DNA or enzyme. These results suggest that: (i) diminaz probably inhibits the bacterial polymerases by direct action on the enzyme, (ii) diminaz does not abolish the template activity of DNA for the bacterial enzymes, and (iii) the mechanism of inhibition of the bacterial vs. the mammalian enzymes may be different. **Effect of Diminazene on RNA Polymerase:** The characteristics of the RNA synthesizing system used have been described (Chamberlain and Berg, 1962). When diminaz (50 µg/ml) was added to the standard reaction mixture, only a slight (<5%) inhibition of RNA synthesis was observed. At 100 µg/ml RNA synthesis was inhibited by 20%. These data indicate that the bacterial RNA polymerase (E. coli) is much less sensitive to inhibition than are the DNA polymerases studied.
DISCUSSION

The observation that the number of trypanosomes in blood does not increase after drug treatment suggests that diminaz inhibits cell division in vivo. This suggestion is supported by ultrastructural alterations seen in trypanosomes exposed in vivo to a curative dose of diminaz and by the near doubling of the DNA, RNA and protein content in these cells. There have been no reports suggesting that diminaz acts to inhibit cytokinesis.

The recovery of $^3$H-thymine from 70% HClO$_4$ hydrolysates of cold dilute acid insoluble material obtained from trypanosomes labeled in vitro or in vivo with $^3$H-TdR, and the alkali stability of the radioactivity in 5% TCA ppt's. indicate that $^3$H-TdR is incorporated into the DNA of the bloodstream forms. Similarly, the chromatographic recovery of $^3$H-cytosine and $^3$H-uracil from PCA hydrolysates indicate that labeled uracil is incorporated into trypanosome RNA.

The reversible inhibition of incorporation of $^3$H-TdR into DNA and of $^3$H-uracil into RNA, under both in vivo and in vitro conditions of drug treatment suggests a decreased permeability to precursor(s) or a decreased rate of nucleic acid synthesis and/or enhanced rate of degradation. The finding of 2X the amount of DNA/cell after drug treatment and inhibition of DNA polymerase reactions in vitro suggest that inhibition of incorporation of label into DNA reflects inhibition of synthesis. In the case of RNA metabolism the apparent absence of drug effect on the E. coli RNA polymerase reaction may indicate that diminaz does not directly affect RNA polymerase in vivo. The isolation of DNA and RNA polymerases from bloodstream trypanosomes should help clarify the effect of trypanocidal aromatic diamidines on these enzymes.
We have attempted to examine the possibility of an enhanced rate of degradation of RNA following drug treatment by using washed trypanosomes in vitro in pulse and chase experiments. These data are to be interpreted cautiously since the RNA synthesized in the presence of diminaz, either in vitro or in vivo, may be qualitatively different from the RNAs synthesized in vivo in the absence of drug. Also, there are quantitative differences in the conversions of uracil in trypanosomes in vivo compared to washed trypanosomes in vitro. The pulse and chase data show that pre-existing RNA is degraded more rapidly in the presence of diminaz than in the absence of diminaz and that RNA synthesized in the presence of drug is more stable than RNA synthesized in the absence of diminaz. These findings suggest the following possibilities: (i) RNA synthesized in the presence of diminaz is qualitatively different, i.e., a more stable species of RNA is made, (ii) RNA synthesized during diminaz treatment is chemically altered and more resistant to RNAase, (iii) mRNA transcribed in the presence of diminaz has a higher affinity for ribosomes than normal mRNA and (iv) RNA transcribed in the presence of drug remains attached to the DNA template. The latter possibility could account, in part, for the 30 min lag period before inhibition of $^{14}$C-uracil incorporation was seen (see Fig. 17), i.e. RNA synthesis ceases when all transcription sites are saturated. To distinguish among these possibilities it will be necessary to examine the effects of diminaz on in vitro RNA and protein synthesizing systems, the components of which are derived from bloodstream trypanosomes. The effects of diminaz on trypanosome RNAases should also be examined. Likewise, pulse and chase experiments should be done on bloodstream trypanosomes maintained in vitro in an appropriate culture medium.
Diminaz inhibits the activity of DNA polymerase reactions when *E. coli*, *Micrococcus lysodeikticus* or calf thymus enzymes (pol I) are used, i.e. addition to 100 μg/ml of drug instantaneously stops incorporation of $^3H$-dTTP into DNA. Radioactivity incorporated into DNA prior to addition of diminaz is stable during prolonged (up to 90 min) incubation of the reaction mixture at 37°C in the presence of diminaz. These findings indicate that diminaz does not itself degrade the DNA synthesized nor does it appear to activate the 3'→5' and 5'→3' exonuclease activities of DNA polymerase (Setlow & Kornberg, 1972).

The two bacterial enzymes were more sensitive to diminaz inhibition than was the mammalian enzyme. diminaz inhibition of the bacterial enzyme could be overcome only by the addition of DNA polymerase, indicating action on the enzyme. diminaz inhibition of the mammalian enzyme could be overcome either by addition of template DNA or DNA polymerase. These findings suggest that diminaz acts on DNA polymerase (pol I) and that the calf-thymus enzyme is more sensitive to template-drug interaction than the bacterial enzymes. Should Berenil exhibit differential activity on replicative DNA polymerases from trypanosomes, kinetoplast selectivity may reflect increased drug sensitivity of the mitochondrial enzyme. The progressive disorganization of the KP-DNA seen after diminaz treatment may result from stimulation of exonuclease activity, either that apart from or that associated with a mitochondrial DNA polymerase.

These data do not indicate whether inhibition of DNA (and possibly RNA) synthesis is causally related to the inhibition of
cytokinesis or primary to the trypanocidal action of diminaz on T. brucei. Temporary inhibition of DNA synthesis, perhaps through induction of a state of unbalanced growth, could abolish the synchrony of the cell cycle and render the trypanosomes unable to divide. This could favor either an immunologically mediated elimination of trypanosomes by the host or continued growth until a critical mass to surface ratio is exceeded resulting in cell lysis. Earlier evidence suggests that the host reticulo-endothelial system participates in the chemotherapeutic activity of Antrycide (quinapyramine) and Suramin (Sen et al., 1955). An immune clearing following drug treatment has been suggested to explain the mechanism of action of several anti-cancer agents which inhibit DNA synthesis (Helmstetter, 1971). It would be interesting to note the course of diminaz cure in infected hosts that were immunosuppressed or splenectomized.
Key to Symbols Used in Electron Micrographs

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>cr</td>
<td>cristae</td>
</tr>
<tr>
<td>K</td>
<td>Kinetoplast</td>
</tr>
<tr>
<td>KD</td>
<td>Kinetoplast DNA</td>
</tr>
<tr>
<td>Km</td>
<td>Kinetoplast membrane</td>
</tr>
<tr>
<td>M</td>
<td>mitochondrion</td>
</tr>
<tr>
<td>Mt</td>
<td>subpellicular microtubles</td>
</tr>
<tr>
<td>N</td>
<td>nucleus</td>
</tr>
<tr>
<td>n</td>
<td>nucleolus</td>
</tr>
<tr>
<td>nf</td>
<td>nucleolar fragments</td>
</tr>
<tr>
<td>Nm</td>
<td>nuclear membrane</td>
</tr>
<tr>
<td>P</td>
<td>Pellicle</td>
</tr>
</tbody>
</table>
Figure 1: Longitudinal section of an untreated bloodstream form of *T. brucei* (monomorphic) showing nucleus, nucleolus, nuclear membrane with nuclear ribosomes, and daughter kinetoplasts containing the filamentous, rod-like KP-DNA. x 19,000

Figure 2: Longitudinal section of *T. brucei* showing nucleus, nucleolus, and nuclear membrane with nuclear ribosomes. The pellicle (glycoprotein coat), cell membrane and underlying microtubules can be seen. x 19,000

Figure 3: Longitudinal section of untreated *T. brucei* showing the kinetoplast and KP-DNA. The continuity of the kinetoplast and mitochondrion can be seen. x 56,000

Figure 4. Longitudinal section of untreated *T. brucei* showing the kinetoplast and KP-DNA. The double membrane of the kinetoplast is visible and cristae-like structures are seen in the kinetoplast matrix. x 54,800

Figures 5a and 5b: Longitudinal sections of *T. brucei* exposed in vivo for four hours to a curative drug dose. The filamentous, rod-like KP-DNA is fragmented into globular masses.

a) x 48,000

b) x 26,600
Figure 6: Longitudinal section of *T. brucei* exposed in vivo for four hours to a curative drug dose showing nucleus and nucleolus. x 17,850

Figure 7a, b, c: Longitudinal sections of *T. brucei* exposed in vivo for eighteen hours to a curative drug dose. KP-DNA appears fragmented, as in Figs. 5a and 5b. Nucleolar condensation or fragmentation is evident.
   a) x 7580
   b) x 8660
   c) x 6500

Figure 8: Comparison of serum levels of $^3$H-thymidine in diminaz treated and untreated mice infected with *T. brucei*. At zero time, three mice were injected with either buffer 2 (0.2 ml.) or Berenil (10 µg/g) dissolved in buffer 2 (0.2 ml.) 30 minutes later, all animals were injected with $^3$H-thymidine (0.5 mCi/0.25 ml.). Tail blood samples were taken at 0.5, 1, 2, 3, and 4 hours after $^3$H-thymidine injection and the sera obtained by centrifugation. Aliquots of sera were spotted on Millipore filters and counted by the liquid scintillation method.

Figure 9: Comparison of serum levels of $^3$H-uracil in diminaz treated and untreated mice infected with *T. brucei*. Same as Fig. 8 except that diminaz treated and control animals were injected with $^3$H-uracil (0.5 mCi/0.25 ml.) one hour after injection of Berenil or buffer. Tail blood samples were taken at 0.5, 1, 2.5, and 4 hours after $^3$H-uracil injection.
Figure 10: Comparison of $^3$H-thymidine incorporated into the DNA of *T. brucei* in diminaz. treated and untreated mice. At zero time, control animals were injected with 0.2 ml. buffer 2 and experimental animals received 0.2 ml. diminaz (10 µg/g). 30 minutes later all animals were injected with $^3$H-thymidine (0.3 mCi/0.3 ml.). Blood was harvested from control and experimental animals 0.5, 1.5, and 3.0 hours after $^3$H-thymidine injection. Trypanosomes were purified from mouse blood components and counted. Aliquots containing $10^7$ trypanosomes were precipitated with cold 5% TCA. The precipitates were filtered and counted by the liquid scintillation method. Each point on the curve represents the average of values obtained from three animals.

Figure 11: Relative amount of $^3$H-thymidine incorporated into trypanosome DNA at various times after treatment with diminaz. *in vivo*. At zero time, control animals were injected with buffer 2 (0.2 ml) and experimental animals were injected with diminaz (10 µg/g, 0.2 ml). At 30 minutes, 4 hours, and 5 hours after injection of buffer or diminaz, animals were injected with $^3$H-thymidine (0.3 mCi/0.3 ml). After two hours of exposure to the isotope, trypanosomes were harvested and purified. Triplicate samples from different animals, containing $10^7$ cells, were precipitated with cold 5% TCA, filtered on Millipore filters and counted. All controls showed the same level of incorporation (100%) whether $^3$H-TdR was given 0.5, 4 or 5 hours after injection with buffer.
Figure 12: Comparison of $^3$H-uracil incorporated into RNA of *T. brucei* in diminaz-treated and untreated mice. Same as Fig. 10 except that $^3$H-uracil (0.2 mCi/0.2 ml.) was administered to all mice one hour after injection with diminaz or buffer. Blood was harvested from control and experimental animals 1, 3, 5, and 7 hours after injection with $^3$H-uracil.

Figure 13: Relative amount of $^3$H-uracil incorporated into trypanosome RNA at various times after treatment with diminaz *in vivo*. Same as Fig. 11 except that $^3$H-uracil (0.2 mCi/0.2 ml) was administered 1 hour and 3 hours after injection with buffer or diminaz, and trypanosomes were harvested 1 hour after administration of $^3$H-U. (When trypanosomes were harvested 3 hours after administration of $^3$H-U, exactly the same curve was obtained). All controls showed the same level of incorporation (100%) whether $^3$H-U was given 1 or 3 hours after injection with buffer.
TABLE I

Effect of Diminaz on DNA, RNA, and protein content of *T. brucei*

<table>
<thead>
<tr>
<th></th>
<th>DNA (µg/cell)</th>
<th>RNA (µg/cell)</th>
<th>Protein (µg/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated Trypanosomes</td>
<td>$1.7 \times 10^{-7}$</td>
<td>$8.6 \times 10^{-7}$</td>
<td>$60.7 \times 10^{-7}$</td>
</tr>
<tr>
<td>Trypanosomes from Berenil treated mice (10µg/g; 12 hrs.-2.2 generation times)</td>
<td>$3.0 \times 10^{-7}$</td>
<td>$20.4 \times 10^{-7}$</td>
<td>$120.9 \times 10^{-7}$</td>
</tr>
</tbody>
</table>

DNA was determined by the p-nitrophenylhydrazine method (Webb and Levy, 1955). RNA was determined by the orcinol method (Schneider, 1937). Protein content was determined by the Lowry method.
Figure 14: Effect of diminaz on Incorporation of $^3$H-TdR into DNA at 25°C in vitro. DEAE-purified trypanosomes isolated from untreated mice (controls) and diminaz treated mice (Berenil in vivo, 10 µg/g) were suspended in buffer 2 at a concentration of $10^7$ cells/ml. and warmed to 25°C. Twenty-five ml. aliquots of control cells were incubated without diminaz and with diminaz (at 10 µg/ml and 20 µg/ml) and twenty-five ml. aliquots of trypanosomes from diminaz treated mice were incubated without diminaz and with diminaz (10 µg/ml). diminaz was added to flasks 1 minute before addition of $^3$H-thymidine (4 µCi/ml). Duplicate 0.5 ml. aliquots were removed at various times after isotope was added and precipitated with cold 5% TCA. TCA precipitates were filtered and counted by the liquid scintillation method.

Figure 15: Effect of diminaz on incorporation of $^3$H-TdR into DNA at 37°C in vitro. DEAE-purified trypanosomes from untreated mice were suspended in buffer 2 at a concentration of $10^7$ cells/ml. and warmed to 37°C. Twenty-five aliquots of cells were incubated in the absence of diminaz and in the presence of diminaz (2 µg/ml and 5 µg/ml). diminaz was added to flasks 1 minute prior to addition of $^3$H-thymidine (4 µCi/ml). Duplicate 0.5 ml. aliquots were removed at various times after addition of $^3$H-thymidine, precipitated with cold 5% TCA and counted.
Figure 16: Reversibility of diminaz inhibition of $^{3}$H-TdR incorporation at 37°C in vitro. DEAE-purified trypanosomes from untreated mice were suspended in buffer 2 at a concentration of 10^7 cells/ml and incubated at 37°C for 30 minutes in the presence of 10 μg/ml diminaz. The trypanosomes were removed from the suspending medium by centrifugation and resuspended in buffer 2 without diminaz containing $^{3}$H-thymidine (4 μCi/ml). Cell samples were removed and counted as in Fig. 14.

Figure 17: Effect of diminaz on incorporation of $^{14}$C-uracil into RNA at 25°C in vitro. Same as Fig. 14, except that trypanosomes were labeled with $^{14}$C-uracil (1.2μCi/ml).

Figure 18: Effect of diminaz on incorporation of $^{14}$C-uracil into RNA at 37°C in vitro. Same as Fig. 19, except that trypanosomes were labeled with $^{14}$C-uracil (0.6μCi/ml).
Figure 19a: \(^{14}\text{C-uracil pulse and chase, 5 minute pulse, 25°C. DEAE-purified trypanosomes from untreated mice were suspended in buffer 2 at a concentration of } 10^7 \text{ cells/ml and warmed to 25°C. Twenty-five ml. aliquots of cells were used. The pulse (}^{14}\text{C-uracil, 0.66μCi/ml)} \text{ was given in the presence and absence of diminaz (3.3μg/ml), and the chase was performed (by addition of a 1000 fold excess of unlabeled uracil) in the presence and absence of diminaz (3.3 μg/ml). The pulse and chase were performed in the following manner:}

(1) 5' Pulse (minus diminaz)-chase (minus diminaz)
(2) 5' Pulse (minus diminaz)-chase (plus diminaz)
(3) 5' Pulse (plus diminaz)-chase (plus diminaz)

Duplicate 0.5 ml. aliquots of cells were removed at the conclusion of the pulse and at various times after the chase, precipitated with 5% TCA, filtered and counted.

Figure 19b: \(^{14}\text{C-uracil pulse and chase, 45 minutes pulse, 25°C. Same as Fig. 19a, except that pulse time was 45 minutes.}

Figure 19c: \(^{14}\text{C-uracil pulse and chase, 5 minute pulse, 37°C. Same as Fig. 19a, except at 37°C.}

Figure 19d: \(^{14}\text{C-uracil pulse and chase, 45 minute pulse, 37°C. Same as Fig. 19a, except that pulse time was 45 minutes at 37°C.}
Figure 20: Effect of diminaz on incorporation of $^{14}$C-Leu into protein at 25°C in vitro. Same as Fig. 15, except that trypanosomes were labeled with $^{14}$C-leucine (0.4μCi/ml), and incubated at 25°C with and without diminaz (10 μg/ml and 20 μg/ml).

Figure 21: Effect of diminaz on incorporation of $^{14}$C-Leu into protein at 37°C in vitro. Same as Fig. 20, except that trypanosomes were incubated at 37°C with and without diminaz (2μg/ml and 5μg/ml).
Figure 22: Radioactivity profile obtained from paper chromatogram of PCA hydrolysates of trypanosomes labeled with $^3$H-thymidine in vivo (2 hours, 0.5 mCi/mouse) and in vitro (1 hour, 37°C, 4μCi/ml, $10^7$ cells/ml buffer 2). The chromatogram was developed for 24 hours at room temperature in solvent 1.

Figure 23: Radioactivity profile obtained from paper chromatogram of PCA hydrolysate of trypanosomes labeled in vivo with $^3$H-uracil (2 hours, 0.5 Ci/mouse). The chromatogram was developed for 24 hours at room temperature in solvent 1.

Figure 24: Radioactivity profile obtained from paper chromatogram of PCA hydrolysate of trypanosomes labeled in vitro with $^{14}$C-uracil (1 hour, 25°C, 0.66μCi/ml, $10^7$ cells/ml in buffer 2). The chromatogram was developed for 24 hours at room temperature in solvent 1.

Figure 25: Radioactivity profile obtained from paper chromatogram of PCA hydrolysate of trypanosomes labeled in vitro with $^{14}$C-uracil. Same conditions as Fig. 24, except labeling was performed at 37°C.
TABLE II
Location of Radioactivity in Two Dimensional Chromatograms

<table>
<thead>
<tr>
<th>Base</th>
<th>Radioactivity from cells labeled with $^3$H-U (cpm)</th>
<th>Radioactivity from cells labeled with $^3$H-TdR (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>47</td>
<td>43</td>
</tr>
<tr>
<td>A</td>
<td>34</td>
<td>38</td>
</tr>
<tr>
<td>C</td>
<td>368</td>
<td>46</td>
</tr>
<tr>
<td>U</td>
<td>380</td>
<td>35</td>
</tr>
<tr>
<td>T</td>
<td>61</td>
<td>315</td>
</tr>
<tr>
<td>Base</td>
<td>$R_f$ value in Solvent 1</td>
<td>$R_f$ value in Solvent 2</td>
</tr>
<tr>
<td>------</td>
<td>-------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>G</td>
<td>0.17</td>
<td>0.19</td>
</tr>
<tr>
<td>A</td>
<td>0.30</td>
<td>0.43</td>
</tr>
<tr>
<td>C</td>
<td>0.46</td>
<td>0.45</td>
</tr>
<tr>
<td>U</td>
<td>0.70</td>
<td>0.53</td>
</tr>
<tr>
<td>T</td>
<td>0.79</td>
<td>0.63</td>
</tr>
</tbody>
</table>
Figure 26: Time dependence of the three DNA polymerase reactions. All polymerases were present at a concentration of 5 U/ml in the standard reaction mixture. [DNA] = 88 µg/ml.

Figure 27: The effect of increasing concentrations of diminaz on the DNA polymerase catalyzed incorporation of $^3$H-dTTP into DNA. All polymerases were at a concentration of 5 units/ml in the standard reaction mixture. [DNA] = 176 µg/ml.

Figure 28: Conditions for reversibility of diminaz inhibition of E. coli DNA polymerase. The effect of adding polymerase, DNA and sRNA to the standard reaction mixture initially containing 5 µg/ml Berenil, 5 units/ml E. coli DNA polymerase and 176 µg/ml DNA was examined.

Figure 29: Conditions for reversibility of diminaz inhibition of M. lysodeikticus DNA polymerase. The effect of adding polymerase and DNA to the standard reaction mixture initially containing 5 µg/ml diminaz, 5 units/ml M. lysodeikticus DNA polymerase and 176 µg/ml DNA was examined.

Figure 30: Conditions for the reversibility of diminaz inhibition of calf thymus DNA polymerase. The effect of adding polymerase, DNA and sRNA to the standard reaction mixture initially containing 10 µg/ml diminaz, 5 units/ml calf thymus DNA polymerase and 176 µg/ml DNA was examined.
Literature Cited


Brown, D. G. and D. S. Coffey 1972. Effects of polyinosinic acid and polycytidylic acid on the deoxyribonucleic acid template activity of isolated nuclei and soluble chromatin from rat liver. J. Biol. Chem. 247, 7674.


Protein measurement with the Folin phenol reagent. J. Biol. 
Chem. 193, 265.


Average of six trials:
3 controls, 3 Berenil treated animals. Berenil or buffer administered I. P. 30' prior to \textsuperscript{3}H-Tdr injection

- deviations

CPM/\mu l serum

Time After I. P. Isotope Injection
Average of six trials: 3 Berenil treated animals, 3 Berenil or buffer administered I.P. thr. prior to H-U injection. Extremes of deviations.

3 controls. 3 Berenil treated animals. Berenil or buffer administered I.P. thr. prior to H-U injection.
Figure 11

Percent $^3$H-Tdr Incorporated (relative to Control Cells)

Time (After Berenil Treatment) of $^3$H-Tdr Injection
FIG. 12

CPM/10^7 cells in TCA sol.

Controls

Berenil (10 \( \mu \)g/g)

Time after I.P. Isotope Injection (hrs.)
Control Cells

- - Controls cells + 10μml
- - Berenil + 20μml: in vitro
- - and cells treated with
- - Berenil in vivo (10mg/ kg)
- - and resuspended in vitro
- - with or without Berenil.
FIG 15

Graph showing CPM/10^6 cells in TCA ppt.

- Control
- 2 x 10^3 Bovend
- 5 x 10^3 Bovend

TIME (hrs)

CPM/10^6 cells in TCA ppt.
Relief of Berenil Inhibition of DNA Synthesis at 37°C in vitro

![Graph showing relief of Berenil inhibition of DNA synthesis at 37°C in vitro.](image)

**CPM/10^7 Cells in TCA ppt.**

**Time (hours)**
5' Pulse & 25°C

- 5' Pulse-Chase
- 5' Pulse-Chase in presence of Berenil
- 5' Pulse in presence of Berenil-chase in presence of Berenil

CPM / 10^7 Cells in TCA ppt.

Time after chase (min.)

FIG. 14A
Fig. 19b

45' Pulse @ 25°C

CPM/10^6 Cells in TCA ppt.

- 45' Pulse-chase
- 45' Pulse-chase in presence of Berenil
- 45' Pulse in presence of Berenil-chase in presence of Berenil

Time after chase (min.)
$5'$ Pulse @ $37^\circ$C

- $5'$ Pulse-chase
- $5'$ Pulse-chase in presence of Berenil
- $5'$ Pulse in presence of Berenil-chase in presence of Berenil

CPM/$10^7$ Cells in TCA ppt.

Time after chase (min.)
45' Pulse @ 37°C

CPM/10^7 Cells in TCA ppt.

- 45' Pulse-chase
- 45' Pulse-chase in presence of Berenil
- 45' Pulse in presence of Berenil-chase in presence of Berenil

Time after chase (min.)
Effect of Berenil on Protein Synthesis at 25° in vitro

FIG. 20

Effect of Berenil on Protein Synthesis at 25° in vitro

CPM in TCA ppt./10^6 cells

μC/ml

TIME (min)

10 20 30 40 60 90

10,000

20,000

30,000

40,000

Control

Berenil

Berenil

Berenil
Effect of Berenil on Protein Synthesis at 37° in vitro

![Graph showing the effect of Berenil on protein synthesis at 37° in vitro.](image)
Fig. 23

Fraction Number

CPM

1,500

1,000

500

G

C

U

T

0

10

20

30

40

100
Cells labeled in vitro at $37^\circ$C with $^{14}$C-Uracil

**Fig. 25**
Fig. 29

[Graph showing data with axes labeled: 0-100 on the vertical axis and 10-40 on the horizontal axis.](image)
Fig. 30

[Graph showing data with axes labeled: (photocells ATP incorp./30)].

- [X-axis]: 10, 20, 30 (values in units are not clearly visible)
- [Y-axis]: 0, 10, 20 (values in units are not clearly visible)

Lines on the graph are labeled: JNNA, DNA, RNA.