ZAHALSKY, Marilyn S. Lantz, 1945-
EFFECT OF BERENIL ON NUCLEIC ACID SYNTHESIS IN TRYPANOSOMA BRUCEI.

The City University of New York, Ph.D., 1974
Biochemistry

University Microfilms, A XEROX Company, Ann Arbor, Michigan

EFFECT OF BERENIL ON NUCLEIC ACID SYNTHESIS IN *TRYPANOSOMA BRUCEI*

by

MARILYN LANTZ ZAHALSKY

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

1974

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

1/26/74.
date

Chairman of Examining Committee
Prof. J. F. Hogg

1-26-75
date

Executive Officer

Prof. B. Tropp

Prof. P. D'Alesandro

Prof. S. H. Hutner

Supervisory Committee

Abstract

EFFECT OF BERENIL ON NUCLEIC ACID SYNTHESIS IN *TRYPANOSOMA BRUCEI*

by

Marilyn Lantz Zahalsky

Advisor: Professor James F. Hogg

Previous studies have indicated that the trypanocidal drug Berenil binds to a variety of DNA's and RNA's *in vitro* and inhibits KP-DNA synthesis in culture forms of *Trypanosoma mega*. The present study was undertaken to examine the effect of Berenil on growth and nucleic acid synthesis in bloodstream forms of *Trypanosoma brucei*. The effect of Berenil on nucleic acid synthesis was examined on trypanosomes in the mouse bloodstream (*in vivo*), and on washed trypanosomes *in vitro*. Using purified bacterial and mammalian polymerases, we have examined the effect of Berenil on *in vitro* DNA and RNA synthesizing systems.

Our results indicate that Berenil interferes with the growth of *T. brucei*. Berenil-treated trypanosomes persist in the bloodstream for some time after drug treatment, slowly decrease in number and eventually disappear. Berenil treatment of infected mice results in an accumulation of what appear to be predivisional forms of trypanosomes. These cells contain approximately twice the amount of DNA, RNA and protein usually found in untreated trypanosomes.

Berenil reversibly inhibits the incorporation of labeled thymidine and uracil into DNA and RNA respectively, whether trypanosomes are labeled *in vivo* or *in vitro*. Berenil inhibits the activity of a variety of DNA polymerases, but does not inhibit the activity of *E. coli* RNA polymerase at chemotherapeutically useful concentrations.

We conclude that Berenil inhibits DNA synthesis and interferes with RNA metabolism (either by inhibiting synthesis or enhancing degradation) in trypanosomes. In addition, we conclude that Berenil blocks cell division in *Trypanosoma brucei*.

ACKNOWLEDGEMENTS

These studies were begun at Queens College, C.U.N.Y. in 1969 and completed during 1973 in the Parasitology and Biochemistry Research Laboratories of the Department of Biological Sciences at Southern Illinois University, Edwardsville. In the period between 1969 and 1973 additional practical experience with the African trypanosomiasis was gained as Visiting Investigator at the East African Trypanosomiasis Research Organization, Tororo, Uganda.

It is a pleasure to acknowledge the contributions of the following scientists and staff members whose discussions, assistance, interest and helpful suggestions have contributed to the biochemical approaches taken during the course of my investigations:

Dr. James F. Hogg------for his interest and friendship and because as my first Professor of Biochemistry he motivated me to pursue a career in Biochemistry,

Dr. Burton Tropp------for his skillful insights and practical suggestions in the field of nucleic acid biochemistry and because as instructor and friend he offered many helpful suggestions,

Dr. Aaron Lukton------for his patience and administrative assistance as Executive Officer of the Ph.D. program in Biochemistry,

Dr. Seymour Hutner------for the versatility of his ideas and imaginative resource and because his grasp of the field of Protozoology was extremely helpful,

Dr. William Trager------for his helpful discussions and comments and because his insights as a parasitologist have guided portions of these studies,

Dr. Peter J. Walker------for providing practical and useful experience with the African trypanosomes during my visit to Uganda,

Dr. Adriane Njogu-----for making available the facilities of the Biochemistry Laboratories at the East African Trypanosomiasis Research Laboratories,

Dr. Annette Baich------for her friendship, for her daily illustration of the scientific method, and for her critical insight on the nature of evidence,

Dr. David Brown------for helpful discussions on the polymerases and because some portions of this work were conducted in his laboratory, and finally, Dr. Arthur Zahalsky------whose resources of patience, hard work, teaching and daily activities at the bench provided the motivation and intellectual atmosphere for the work accomplished in this thesis and because his faith in me as scientist, colleague and wife strengthened my conviction that the work being done was of some fundamental importance to the Biochemistry of Parasites.

I also wish to acknowledge the excellent technical assistance of Ms. Ursula Behrens, who kindly provided training in electron microscopy, and the excellent technical assistance of Ms. Valerie K. Pontius who was willing to work at the odd hours that many of the experiments demanded.

To the ladies (Ms. Alice Miller and Ms. Lynn Youngman) who looked after our daughter Maya these past two years, I offer my profound thanks for their kindness, generous spirit and patience.

Finally, to Maya I offer my thanks because of the wonderful little person she is and because her happy little smile made me laugh when my days in the lab were less than ideal.

LIST OF ABBREVIATIONS

Ber	=	Berenil
CT DNA	=	Calf Thymus DNA
CT DNA polymerase	=	Calf Thymus DNA polymerase
IP	=	intraperitoneal
PCA	=	perchloric acid
TCA	=	trichloroacetic acid
Tdr	=	thymidine
U	=	uracil

TABLE OF CONTENTS

LIST OF TABLES AND DIAGRAM

LIST OF FIGURES

Biologists work very close to the frontier between bewilderment and understanding. Biology is complex, messy and richly various, like real life; it travels faster nowadays than physics or chemistry (which is just as well, since it has so much farther to go), and it travels nearer to the ground.

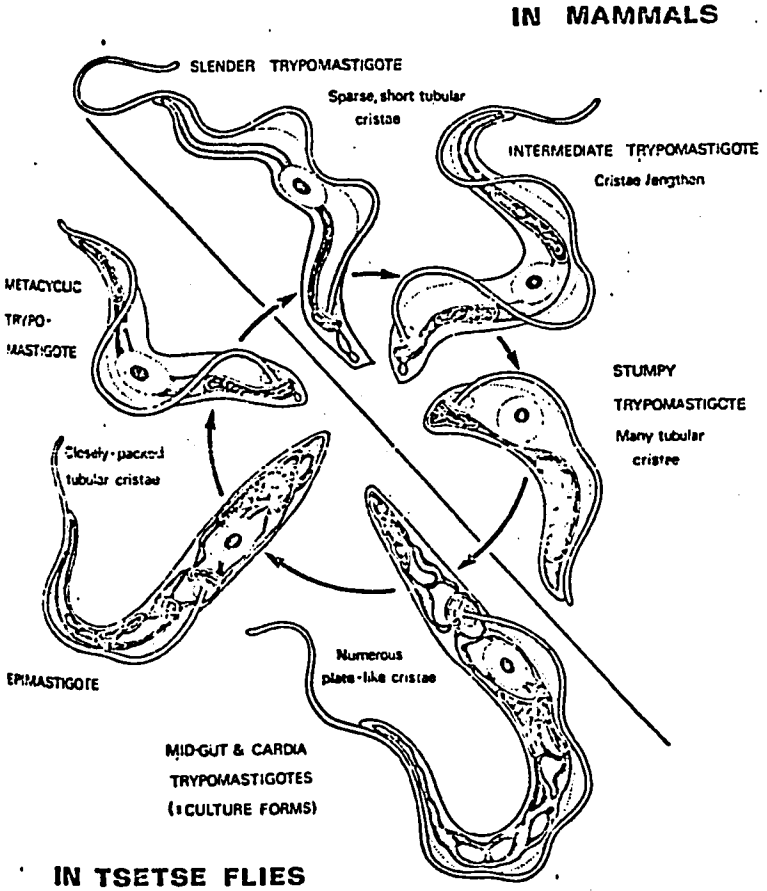
Sir Peter B. Medawar

INTRODUCTION

The trypanosomes of human sleeping sickness, Trypanosoma gambiense and Trypanosoma rhodesiense, are at all times morphologically indistinguishable from Trypanosoma brucei which is responsible for sleeping sickness (nagana) in wild game and domestic animals in Africa. T. brucei does not infect man (Hoare, 1970).

These flagellates, previously designated as the T. brucei subgroup, and now assigned to the subgenus Trypanozoan by Hoare (1964), differ from other tsetse-borne trypanosomes in two important respects: (1) They undergo a complex life-cycle, involving transformation in the midgut and salivary glands of the tsetse fly and (2) they may show a wide variation in morphologic appearance in the mammalian bloodstream, ranging from long slender flagellates with a free flagellum at the anterior end, to short stumpy forms with no free flagellum (Vickerman, 1965).

Life Cycle Diagram



(Vickerman, 1971)

Because of this second feature, bloodstream trypanosomes are referred to as "pleomorphic" or "polymorphic" trypanosomes. Morphological variation is continuous in pleomorphic infections and appears to follow a regular pattern; slender forms predominate when the parasitemia is rising and stumpy forms predominate when the parasitemia is on the decline (Vickerman, 1965). Since polymorphic infections can develop in animals infected with a single trypanosome (Oehler, 1914) polymorphism cannot be the result of a mixed population of genetic variants but rather must result from changes in the morphology of individual trypanosomes i.e. slender forms appear to transform into stumpy forms (Ashcroft, 1957).

Polymorphism is a variable character since polymorphic trypanosomes maintained by syringe passage in laboratory animals become monomorphic, slender forms only being found in the bloodstream. This loss of polymorphism is accompanied by loss of infectivity for tsetse flies (Ashcroft, 1960) and inability to grow in culture (Reichenow, 1932). It is the stumpy forms that undergo transformation and continue to transform upon entering the tsetse fly midgut (Reichenow, 1921; Wijers and Willett, 1960; Rudzinska and Vickerman, 1968) and it is the stumpy forms that undergo development and growth in culture media (Reichenow, 1932). The forms assumed in culture are morphologically and physiologically identical with those found in the tsetse midgut (Vickerman, 1962).

It is not known what elicits the transformation of slender forms into stumpy forms. It has been suggested that host antibody stimulates this transformation (Ashcroft, 1957) however, this appears not to be the case (Balber, 1972). In chronic infections the number of trypano-

somes in the blood fluctuates, each peak of parasitemia exhibiting a different antigenic type (serotype). Antigenic variation occurs in both polymorphic and monomorphic infections and the pattern of antigenic variation can be altered by specific host antibody (Gray, 1962). Variant specific antigens appear to be associated with the glycoprotein surface coat of bloodstream trypanosomes (Vickerman and Luckins, 1969; Allsopp, Njogu and Humphryes, 1971) and also appear to be trypanosomal in origin (Vickerman, 1971; Steiger, 1971). Vickerman (1962) has suggested that the slender-stumpy transformation may in part result from functional and morphological changes that occur in the single, giant mitochondrion of T. brucei and allied species.

The mitochondrion of Trypanosoma brucei undergoes cyclical activation and repression during the life cycle of the organism (see life cycle diagram). The respiration of monomorphic and pleomorphic bloodstream forms of T. brucei is cyanide insensitive. Monomorphic bloodstream forms appear to lack a functional Krebs cycle and a cytochrome-mediated electron transport system (Fulton and Spooner, 1959; Grant and Sargent, 1960, 1961, Grant et al., 1961). These cells metabolize glucose to pyruvate which is excreted into the host bloodstream. They produce negligible carbon dioxide and their energy demands appear to be satisfied by ATP generated during aerobic glycolysis. NAD reduced during glycolysis is oxidized via a non-phosphorylating glycerophosphate oxidase-dehydrogenase system (Grant et al., 1961). This coupled system accounts for the high oxygen demand of bloodstream forms. Morphologically, the mitochondrion of these forms appears as a narrow tubular structure (called a promitochondrion) extending the length of the organism. Internally, the promitochondrion shows few of the cristae which are characteristic of

an active mitochondrion (Vickerman, 1962, 1965, 1971).

Recent work has suggested that some mitochondrial activity is present in pleomorphic strains (Bowman and Flynn, 1968; Flynn and Bowman, 1970). Intermediate and short stumpy forms maintain their motility in buffer, using α-ketoglutarate as substrate, long after movement ceases in slender forms and monomorphic strains (Ryley, 1966; Vickerman, 1965, 1971). In a pleomorphic strain of T. rhodesiense Bowman and Flynn (1968) found significantly lower pyruvate production and significantly higher carbon dioxide and succinate production compared to monomorphic strains, besides utilization of α-ketoglutarate. They concluded that oxidative decarboxylation mechanisms are active in pleomorphic strains but that the Krebs cycle operates at a rate which is quantitatively insignificant in vivo. As yet no cytochromes have been detected in pleomorphic strains (Vickerman, 1971). Flynn and Bowman (1970) suggest that the L-α-glycerophosphate oxidase-dehydrogenase system of bloodstream forms might be augmented by an autooxidizable flavoprotein in the terminal respiration of those trypanosomes that have developed a functional Krebs cycle, i.e. presumably the intermediate and short stumpy forms.

Fly midgut forms and their counterparts - the culture forms - have a mitochondrion that contains a functional Krebs cycle and a cytochrome-mediated electron transport system. Respiration in these forms is cyanide sensitive and glucose is metabolized to carbon dioxide and water (Ryley, 1956, 1962; Fulton and Spooner, 1959). Oxidative phosphoryation accompanies electron transport along the cytochrome chain (Vickerman, 1971). The mitochondrion in these forms is an elaborate network of

kinetoplast-connected canals furnished with numerous plate-like cristae (Vickerman, 1962). The energy yielding metabolism of trypanosomes has been elucidated and correlates with the morphological differences observed in the mitochondrion at various stages in the trypanosome life cycle.

The mechanism of cyclical activation and repression of the trypanosome mitochondrion is unknown (Hill and Anderson, 1970). Activation apparently commences in the bloodstream (slender to stumpy transformation) and repression apparently occurs in the fly salivary gland stages (Vickerman, 1971). The suggestion has been made that KP-DNA is involved in the process of mitochondrial activation and repression (Steinert, 1960).

Although the energy yielding metabolism of trypanosomes has been elucidated, little is as yet known about macromolecular synthesis (especially DNA, RNA and protein synthesis) or the regulation of macromolecular synthesis in these organisms. The nuclear division process of trypanosomes is not well understood, and apparently is not closely akin to eukaryotic mitosis (Vickerman and Preston, 1970). During cell division in T. rhodesiense the nuclear envelope and nucleolus-like endosome persist and become elongated. An acentric spindle of microtubules encases the elongating endosome. As division proceeds the nucleolar material fragments. In bloodstream trypanosomes condensed chromatin (chromosomal material) appears to be peripherally distributed at the nuclear envelope during the phase of nuclear constriction. In culture forms the chromatin is not so abundant. The discrete chromosomes envisaged by microscopists at the light microscope

level in stained preparations of trypanosomes have not been observed in trypanosomes under the electron microscope. There is no evidence for the existence of sexual or parasexual processes in these organisms (Walker, 1964; Vickerman and Preston, 1970). Although the nuclear DNA of these flagellates resembles that of eukaryotes in being histone-bound (Steinert, 1965) there is no evidence suggesting that this DNA is packaged into eukaryote-type chromosomes.

DNA synthesis in bloodstream trypanosomes has not been investigated other than by autoradiography (Ono et al., 1971). DNA polymerases from bloodstream trypanosomes have not been isolated or characterized. Analytical ultracentrifugation in CsCl density gradients of whole cell DNA reveals a main band and one or two satellite bands. In all species of trypanosomes at least one of the satellite bands represents extra-nuclear DNA, the kinetoplast DNA (KP-DNA).

The KP-DNA of trypanosomes is housed in a capsular expansion of the mitochondrion called the kinetoplast. KP-DNA is self-replicating and division of the kinetoplast-mitochondrion complex precedes nuclear division (Vickerman, 1971). KP-DNA represents from 10 to 20% of the total cell DNA of trypanosomes. Electron microscopic examination of purified KP-DNA of several species of trypanosomes has shown it to be composed of small circular and linear molecules. Many of the small circular forms appear to be arranged along a central axis (Riou and Delain, 1969; Simpson and Da Silva, 1971). The KP-DNA of trypanosomes contains the smallest circular microbial DNA (0.5μ contour length) thus far described (Riou and Delain, 1969). KP-DNA differs in base composition from nuclear DNA in having a higher A-T content (Riou and Pautrizel, 1969).

Giemsa-stained preparations of bloodstream populations of all salivarian trypanosomes contain individual organisms that appear to lack a kinetonucleus (KP-DNA). Such trypanosomes lack KP-DNA (Newton and Burnett, 1971) or contain structurally altered KP-DNA (Gutteridge, et al., 1971) and are referred to as dyskinetoplastic trypanosomes (Trager and Rudzinska, 1964). The condition of dyskinetoplasty is not well understood. Although dyskinetoplasty may arise spontaneously, its incidence can be greatly increased by treatment of cells with several compounds i.e. Berenil, acriflavin, ethidium bromide, and p-rosaniline (Rudzinska and Vickerman, 1968). Dyskinetoplasty is irreversible and the condition is inherited by all future progeny. Studies of trypanosome dyskinetoplasty have produced evidence suggesting that complete mitochondrial activation requires intact KP-DNA (Vickerman, 1971). Supporting evidence for this idea is:

(i) Dyskinetoplasty is observed in monomorphic and pleomorphic bloodstream forms; these are viable in the host bloodstream and may be maintained indefinitely by syringe passage in an appropriate host (Stuart, 1971).

(ii) Pleomorphic dyskinetoplastic bloodstream forms do not undergo transformation when placed in culture medium and do not become established in culture (Stuart, 1971). Dyskinetoplastic bloodstream forms do not infect the tsetse fly (Rudzinska and Vickerman, 1968).

(iii) Crithidia grown in the presence of compounds that induce dyskinetoplasty show impaired mitochondrial function, i.e. decreased respiration, decreased mitochondrial enzyme activities and decreased levels of functional cytochromes (see Hill and Anderson, 1970 for review).

RNA synthesis has been studied in trypanosomes by autoradiography

and by observation of the fluxes in total RNA content. Whereas the DNA content remains constant for each trypanosome species, the RNA content varies depending on the growth conditions (*in vivo* vs *in vitro*) (Riou and Pautrizel, 1969). ^{3}H-uridine pulse-labeling experiments have shown that both the kinetoplast and nucleus are sites of RNA synthesis in *Crithidia luciliae* (Steinert et al., 1969). The kinetoplasts of both the culture and bloodstream forms of *T. gambiense* contain RNA (Ozeki et al., 1971).. The sedimentation values and synthesis and processing (precursor-product relationship) of ribosomal RNAs from *Crithidia fasciculata* have been described (M. Gottlieb, Ph.D. thesis 1972) but no such studies have been reported for bloodstream trypanosomes.

Table I summarizes what is known about the mode(s) of action of chemotherapeutic agents currently used to treat the African trypanosomiases (Williamson, 1970). The findings noted in Table I reveal the following:

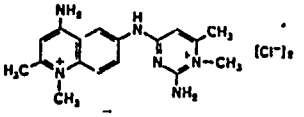
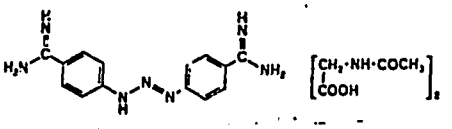
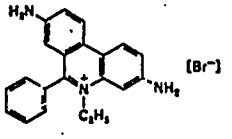
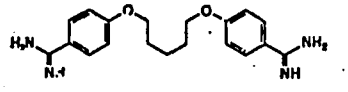
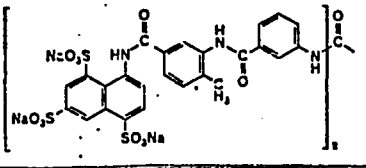
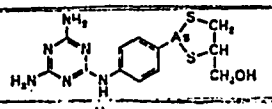
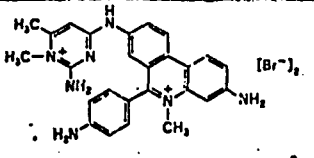
a) Current chemotherapy of trypanosomiasis in man and animals depends on a relatively small group of synthetic drugs.

b) Inferences on the mode(s) of action of existing trypanocidal drugs have been derived almost exclusively from studies using model cell systems, *in vitro* cell-free nucleic acid and protein synthesizing systems, or *in vitro* systems designed to study physico-chemical parameters of drug : nucleic acid interaction.

c) With the exception of Mel B and possibly suramin, these trypanocides are inhibitors of nucleic acid and protein synthesis.

The compounds in use appear to show a specificity of action at two levels: (i) they are selective for certain protozoan parasites within

TABLE 1: Summary of Trypanocidal Agents

Compound: Name	Formula	Actions	References
Antrycide (quinapyramine)		(i) Concentrates in lysosomes (ii) Inhibits incorporation of purines into nucleic acids in Crithidia oncopelti (iii) Causes aggregation of cytoplasmic ribosomes in C. oncopelti (iv) Does not directly inhibit bacterial RNA polymerase	(i) Allison, 1968 (ii), (iv) Newton, 1966 (iii) Elliott, 1963
Berenil (diminazene)		(i) Binds to DNA and RNA in vitro (ii) Selectively inhibits the synthesis of kinetoplast DNA in Trypanosoma mega (iii) Induces dyskinetoplasty	(i) Festy et.al., 1970 (ii) Newton & LePage, 1967; 1968 (iii) Killick - Kendrick, 1964
Ethidium Bromide		(i) Binds to DNA and RNA in vitro (ii) Preferentially inhibits mitochondrial DNA polymerase from rat liver cells (iii) Concentrates in lysosomes (iv) Induces dyskinetoplasty (v) Inhibits bacterial RNA polymerase (vi) Inhibits bacterial DNA polymerase (vii) Inhibits DNA synthesis in C. oncopelti; also inhibits RNA and protein synthesis	(i) Waring, 1970 (ii) Meyer & Simpson, 1969 (iii) Allison, 1968 (iv) Riou, 1967 (v) Waring, 1965 (vi) Elliott, 1963 (vii) Newton, 1957
Pentamidine		(i) Causes fragmentation of nucleolus in Trypanosoma rhodesiense (ii) Inhibits thymidine phosphorylase (iii) Inhibits charging and transfer reactions of protein synthesis in a cell-free system from C. fasciculata (iv) Preferentially fragments kinetoplast DNA but does not directly inhibit bacterial RNA polymerase	(i) MacAdam & Williamson, 1972 (ii) Williamson, 1970 (iii) Kahan et.al., 1968 (iv) Elliott, 1963
Suramin		(i) Concentrates in lysosomes (ii) Inhibits charging and transfer reactions of protein synthesis in a cell-free system from C. fasciculata (iii) Inhibits some lysosomal enzymes (iv) Inhibits bacterial RNA polymerase	(i) Allison, 1968 (ii) Kahan et.al., 1968 (iii) Smeesters & Jacques 1968 (iv) Waring, 1965
Mel B		(i) Generally inhibits Kinases, especially Phosphoenolpyruvate and glycerol kinases	(i) Grant, 1966
P.othidium		(i) Inhibits bacterial RNA polymerase (ii) Induces dyskinetoplasty	(i) Waring, 1965 (ii) Ray & Malhotra, 1960

the host; and (ii) some of them (ethidium bromide, Berenil) selectively inhibit the synthesis of extranuclear DNA in the kinetoplast of trypanosomes or in the mitochondria of other organisms (Newton and Le Page, 1968; Meyer and Simpson, 1969; Delain et al., 1971; Attardi and Attardi, 1971; Mahler and Dawidowicz, 1973).

Berenil (4, 4'-diamidino-diazo-amino-benzene diaceturate) has primarily been used in the treatment of T. congolense and T. vivax infections of cattle. Limited clinical trials carried out in East Africa suggest that the drug may also be of value in the treatment of T. gambiense and T. rhodesiense during early stages of infection, i.e. before central nervous system involvement occurs. Berenil does not pass the blood-brain barrier (Williamson, 1970). In the treatment of cattle trypanosomiasis this drug exhibits a number of advantages over other trypanocidal agents; it is rapidly excreted, Berenil resistant strains rarely occur, and it is active against strains which have become resistant to phenanthridines (Newton, 1972). After intravenous or intraperitoneal injection, the concentration of diamidine drugs (stilbamidine, pentamidine, and Berenil) falls quickly. When 10 mg/kg stilbamidine is injected intravenously into rabbits, the blood concentration is 0.5 μg/ml at 2 hours and less than 0.05 μg/ml at 6 hours. Berenil is excreted quickly and apparently little tissue retention occurs (Hawking, 1963). Walker & Opiyo (1973) have reported that the minimum dose of Berenil needed to cure mice of T. brucei depends among other factors on the level of the parasitemia. Berenil causes few or no chronic toxic effects in man or domestic animals. It is active against some bacteria (Williamson, 1970).

Berenil precipitates a portion of the protein present in cell-free extracts of African trypanosomes (Desowitz, 1960). Berenil binds reversibly to a variety of DNAs and RNAs in vitro, the extent of binding depending directly on the A-T content of the nucleic acid (Newton, 1967; Newton and Le Page, 1968; Festy et al., 1970a, 1970b). Evidence which suggests that the binding of Berenil to DNA is non-intercalative includes the fact that there is: (i) no detectable effect on the viscosity of DNA solutions, and (ii) no detectable uncoiling of the DNA helix as a result of Berenil binding (Newton, 1967; Waring, 1970). No evidence of complex formation between Berenil and mononucleotides has been found (Newton, 1967). In neutral solution the Berenil molecule undergoes a rearrangement which results in the formation of an o-aminoazo derivative of the original triazine structure. This breakdown product has no trypanocidal activity and apparently does not bind to DNA (Newton, 1967). Recent growth inhibition studies on T. mega (Newton, 1972) suggest that the spacing of the amidine groups in Berenil is critical to its trypanocidal activity.

Berenil has been referred to as a "kinetoplast selective" agent by several investigators (Newton, 1967; Newton and Le Page, 1967, 1968; MacAdam and Williamson, 1969, 1972; Brack et al., 1972a, 1972b). The designation of Berenil as "kinetoplast selective" is based on the following observations:

(i) T. mega grown in the presence of Berenil first shows fluorescence in the kinetoplast and only subsequently in the nucleus. When these organisms were grown in the presence of Berenil for a time

sufficient to produce fluorescence in the kinetoplast but not in the nucleus, only the buoyant density of the nuclear DNA was increased following addition of 5-bromodeoxyuridine. Neither the concentration nor time dependence of this effect of Berenil has been reported (Newton and Le Page, 1967).

(ii) When trypanosomes in the host bloodstream are exposed to a curative dose of Berenil for various lengths of time (up to six hours) the KP-DNA is observed to be fragmented whereas the nucleus appears normal (MacAdam and Williamson, 1969, 1972).

(iii) Brack et al., (1972a) have reported that Berenil treatment of culture forms of T. cruzi increases the proportion of branched circles (which are thought by the authors to represent circles in the process of replication) in KP-DNA by a factor of 10^3. The length of the replicated branches is not distributed at random, but into several populations, which corresponded to 15% of the total contour length (0.5 μ). Since they were unable to determine whether untreated KP-DNA contained similar short replication units, they could not determine whether the short replication units seen after drug treatment were induced by Berenil. They concluded that each of the minicircles is able to replicate independently.

The basis for the observed kinetoplast selectivity of Berenil is not understood. It has been suggested (Newton and Le Page, 1968) that the preferential binding of Berenil to A-T base pairs might be a contributing factor, since KP-DNA has a higher A-T content than nuclear DNA. However, ethidium bromide exhibits no base preferences in binding to DNA and it is also kinetoplast selective. It is possible that the lack of histones in KP-DNA makes this DNA more accessible to Berenil than is

the nuclear DNA (Steinert, 1965). This could contribute to the kinetoplast selectivity of the drug. Meyer and Simpson (1969) have isolated both mitochondrial and nuclear DNA polymerases from rat liver cells and have shown that the mitochondrial enzyme is more sensitive to inhibition by ethidium bromide and acriflavin than is the nuclear enzyme. Perhaps the kinetoplast selectivity of Berenil is in part due to its ability to preferentially inhibit KP-DNA polymerase over nuclear DNA polymerase in trypanosomes.

Previous investigations have not explored the mode of action of Berenil on African bloodstream trypanosomes. Though its apparent selectivity for inhibiting KP-DNA synthesis is an interesting property of this drug, this property may not be solely responsible for its mode of action in vivo, especially in view of the fact that the mitochondrion of bloodstreams forms is "inactive," and that drug-induced dykinetoplastic bloodstream trypanosomes are viable (Stuart, 1971).

One result which might be expected from the interaction of Berenil with nucleic acids is inhibition of nucleic acid synthesis and/or protein synthesis. The present study was undertaken to determine whether Berenil inhibits nucleic acid synthesis in trypanosomes growing in the host bloodstream. In addition, we have examined the action of Berenil on a variety of in vitro nucleic acid synthesizing systems. We have also sought to determine whether the effect of Berenil on nucleic acid synthesis in bloodstream trypanosomes is sufficient to account for its ability to cure African trypanosomiasis.

MATERIALS

Radiochemicals

Thymidine-6-^{3}H (^{3}H-Tdr) (spec. act. 9.82 Ci/mmoles), Uracil-6-^{3}H (^{3}H-U) (spec. act. 27.5 Ci/mmole) and L-Leucine-^{14}C (uniformly labeled) (^{14}C-Leu) spec. act. 255 mCi/mmole) were purchased from New England Nuclear, Boston, Mass. Uracil-2,6-^{14}C (^{14}C-U) (spec. act. 115 mCi/mmole) was purchased from Mallinkrodt Chemicals, St. Louis, Missouri. [Methyl-^{3}H]dTTP (^{3}H-dTTP) (spec. act. 75 Ci/mmole) and ^{3}H-5-UTP (^{3}H-UTP) (spec. act. 4 Ci/mmole) 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, spec. act. 5000 U/mg and calf thymus DNA polymerase EC 2.7.7.7, spec. act. 100 U/mg were purchased from General Biochemicals, Chagrin Falls, Ohio. M. lysodeikticus DNA polymerase, EC 2.7.7.7, spec. 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, spec. act. 600 U/mg, DEAE cellulose, highly polymerized calf thymus DNA, yeast sRNA, pyruvate kinase, deoxyribonuclease I (EC 3.1.4.5, spec. act. 2000 Kunit units/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.

Berenil (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.

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 CF1 mice (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

Buffer 1: EDTA 5.0 gm/liter; Trizma base 5.0 gm/liter; D-glucose 2.0 gm/liter; NaCl 4.0 gm/liter; KCl 0.2 gm/liter; adjusted to pH 7.5 with HCl.

Buffer 2: Trizma base 5.0 gm/liter; D-glucose 2.0 gm/liter; NaCl 4.0 gm/liter; KCl 0.2 gm/liter; $CaCl_2$ 0.2 gm/liter; $MgCl_2 \cdot 6H_2O$ 0.2 gm/liter, adjusted to pH 7.5 with HCl.

METHODS

Growth Conditions, Harvesting and Purification of Trypanosomes

Trypanosoma brucei was maintained in CF1 and NLW mice by syringe passage of infected mouse blood. The infection and Berenil cure proceed identically in both strains of mice. Experimental animals were infected by intraperitoneal injection of an appropriate number (1×10^6-2×10^7) of trypanosomes to produce a heavy parasitemia (1-2 x 10^8 trypanosomes/ml blood) in 36 hours. No experimental animals were used longer than 48 hours post-infection. The generation time of this strain in mice is 5-5.5 hours. One infective trypanosome can kill a mouse in seven days. The parasitemia produced by this strain of *T. brucei* in mice runs a fulminating course with the number of parasites in the blood increasing until death of the mouse occurs. The degree of parasitemia was determined by hemocytometry or examination of wet mounts of infected mouse blood.

Trypanosomes were purified from mouse blood cells by differential centrifugation and passage through an anion exchanger as follows: Infected blood was obtained by cardiac puncture and mixed with 5 volumes of cold buffer 1. The entire isolation procedure was carried out at 4°C. The blood-buffer mixture was centrifuged at 365 x g for 10 minutes. At this force the red cells and platelets pack at the bottom of the tube and the trypanosomes settle as a loose white layer on top of the buffy coat. Trypanosomes were resuspended in the supernatant fraction by gentle stirring and the resuspended cells were drawn off and recentrifuged at 1020 x g for 10 minutes. The pellet contained trypanosomes and some

contaminating host blood cells. The 1020 x g pellet was resuspended in buffer 2 and passed through a column of DEAE cellulose (Lanham, 1968) equilibrated with buffer 2. The column was eluted with buffer 2 until the cloudiness dissappeared from the effluent. The eluate, free of host blood cells, contained the purified trypanosomes.

Preparation of Trypanosomes for Electron Microscopy

The trypanosome 1020 x g pellets (either before or after DEAE-cellulose filtration) were fixed in cold 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.4 for one hour. After two rinses in cold 0.1M sodium cacodylate buffer (pH 7.4) the pellets were post-fixed with 1% OsO_4 in the same buffer for one hour. All subsequent steps were carried out at room temperature. The pellets were broken into small pieces and overlayered with 1% aqueous uranyl acetate for 20 minutes, after which the uranyl acetate was withdrawn and the material overlayered with 70% ethyl alcohol. The fragmented pellets 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 hours.

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 minutes followed by 0.4% lead citrate in 0.1M NaOH for 5 minutes.

Electron micrographs were taken with a Jelco TM or a Phillips 300 electron microscope.

Berenil Treatment and Labeling of Trypanosomes

Definitions:

In vivo Berenil treatment and labeling refers to treatment and labeling of trypanosomes during their residence in the mouse bloodstream.

In vitro Berenil treatment and labeling refers to treatment and labeling of whole living trypanosomes purified from mouse blood components and suspended in buffer 2.

In vivo:

Berenil was dissolved in buffer 2 and administered in a small volume (0.2 ml) to mice by intraperitoneal (IP) injection.

Thymidine-6-^{3}H and uracil-6-^{3}H (sterile, aqueous) were administered by IP injection (0.2-0.5 mCi/mouse, in 0.1-0.3 ml).

In vitro:

DEAE purified trypanosomes were suspended in buffer 2 at a concentration of 10^{7} cells/ml. Twenty-five ml. aliquots of cells were placed in 50 ml flasks, warmed to the appropriate temperature and Berenil (100 µl of an appropriate concentration dissolved in buffer 2) was added to the flasks. Two minutes later, ^{3}H-Tdr, ^{14}C-U or ^{14}C-Leu was added to the flasks.

Pulse and chase experiments were performed by exposing the trypanosomes for an appropriate length of time to ^{14}C-U and then adding a 1000 fold excess of unlabeled uracil to the suspending medium as the chase.

Determination of Levels of Isotope in Sera of Mice Injected with ^{3}H-Tdr or ^{3}H-U.

Serum isotope levels in Berenil treated and untreated infected mice were determined as follows: Twenty µl samples of tail blood were collected in heparinized capillary tubes at various times after isotope injection. These capillary tubes were centrifuged in a hematocrit centrifuge and 5 µl samples of serum were 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.

Determination of Radioactivity in Trypanosomes Labeled In Vivo or In Vitro.

(a) Whole cells: 5×10^6 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) Determination of Nucleic acids: 5×10^6 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 of RNA as follows: TCA precipitates as obtained in (b) above were centrifuged at 1465 x g for 15 minutes. The precipitates were washed with 5% TCA and resuspended in 2.5 mls of 0.5 N NaOH. After a 2 hour incubation at 37°C to hydrolyze RNA, the solutions were chilled, and 250 µg of BSA (in 1 ml H_2O) was added. After neutralization with 2.5 mls of 0.5N HCl, 6 mls of cold 10% TCA were added. The TCA precipitates were collected on Millipore filters and

washed several times with 5% TCA (Munro and Fleck, 1966).

(c) Determination of protein: TCA precipitates obtained in (b) above were boiled in 5% TCA for 20 minutes before Millipore filtration (Munro and Fleck, 1966).

All Millipore filters were placed in glass vials, dried and counted in a Nuclear Chicago Mark II liquid scintillation counter using a toluene based scintillation fluid.

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 described above. After being passed through DEAE cellulose, the cells were centrifuged at 1020 x g for 10 minutes and the pellets were resuspended in a final volume of 1.2 ml of buffer 2. To this was added 2 ml of 7% $HClO_4$. The solution was placed on ice for 3 min and the precipitate was centrifuged at 1465 x g for 10 minutes. The $HClO_4$ precipitate was washed twice with a 2% $HClO_4$ solution containing 2.0 x 10^{-3}M sodium pyrophosphate. The washed precipitate was hydrolyzed in 70% $HClO_4$ for 1 hour at 100°C. The hydrolysate was chilled, neutralized with KOH and frozen (Munro and Fleck, 1966). The hydrolysate was taken from the freezer and centrifuged at 1465 x g for 10 minutes to remove precipitated $KClO_4$ from solution. 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 $HClO_4$ hydrolysate and bases 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).

Analysis of Two Dimensional Chromatograms:

The spots corresponding to the five bases were located using a short-wavelength U.V. lamp, and were scraped off the cellulose thin-layer plate. The cellulose powder from each spot was collected and placed in a glass vial. Radioactivity was located by adding a toluene based scintillation fluid to the vials and counting the samples in a Nuclear Chicago Mark II liquid scintillation counter.

Analysis of One Dimensional Chromatograms:

After development in Solvent 1, the chromatograms were dried and the spots corresponding to the five bases were located. The paper was cut into 0.5" strips, and each strip was placed in a glass vial and counted using a toluene based scintillation fluid. The location of the radioactivity was compared with the location of the spots.

DNA, RNA and Protein Determination:

DNA was determined by the PNPH method of Webb and Levy (1955). RNA was determined by the orcinol method (Schneider, 1957). Protein was determined by the method of Lowry (1951). DNA and RNA content were determined on 5% TCA hydrolysates (30 minutes, 100°C) of purified trypanosomes. Protein content was determined using homogenates of purified trypanosomes.

DNA Polymerase Assay:

DNA polymerase was assayed by the filter paper disk technique of Bollom (1966) as described by Brown and Coffey (1972). The standard assay system contained the following constituents in a final volume of 0.1 ml: 10 μmoles of Tris-HCl, pH 7.4, at 37°; 0.7 μmole of $MgCl_2$;

0.1 μmole β-mercaptoethanol; 18.7 nmoles each of dCTP, dGTP, and dATP; 20.0 nmoles of dTTP containing 1μCi of [^{3}H]dTTP; 0.1-0.5 units of appropriate DNA polymerase; 1 to 10 μg of native or heat denatured calf thymus DNA; and an ATP generating system containing 0.25 μmole of ATP, 0.5 μmole of sodium phosphoenolpyruvate, and 0.4 μg of pyruvate kinase. The mixture was incubated at 37° for an appropriate length of time. The reaction was stopped by placing the entire reaction mixture on a filter paper disk at 50°C. The paper disk was dried and washed successively at 4°C as follows: 2 x for 20 min in 5% TCA + 0.01% sodium pyrophosphate; 2 x for 20 min in 5% TCA + 0.01% ATP; 2 x for 5 min in ethanol; 1 x for 2 min in ether. The radioactivity on the disk was determined by liquid scintillation counting using a toluene based scintillation fluid.

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 of Tris-HCl, pH 7.9 at 37°; 0.4 μmoles $MgCl_2$; 0.1 μmole $MnCl_2$; 1.2 μmoles β-mercaptoethanol; 0.04 μmole each of ATP, CTP and GTP; 0.04 μmole UTP containing 0.5 μCi of ^{3}H-5-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° for 10 to 30 minutes. The reaction was stopped by placing the entire reaction mixture on a filter paper disk at 50°C (Bollum, 1966). The paper disk was dried and washed exactly as in the DNA polymerase assay except that in the second washes, 0.01% ATP was replaced by 0.01% UTP. After drying, the radioactivity on the disks was determined by liquid scintillation counting using a toluene based scintillation fluid.

RESULTS

Effect of Berenil on the Growth of Trypanosoma brucei In Vivo.

When a curative dose of Berenil (10μg/g body weight) is administered to a heavily infected mouse ($\sim 10^8$ trypanosomes/ml blood) the trypanosomes do not increase in number but persist in the bloodstream at the same level for some time (generally twelve to eighteen hours). At this time they appear to have normal motility but are larger than trypanosomes from untreated animals. Parasite numbers then begin to decrease, the parasitemia level in the peripheral bloodstream reaching zero within one to four days after treatment. The rate of disappearance of trypanosomes from the peripheral bloodstream of Berenil-treated mice varies slightly with the individual and appears to be directly dependent on the amount of Berenil administered (from 10 to 100 μg/g body weight). The minimum curative dose of Berenil under the conditions used in these studies was found to be approximately 5 μg/g body weight. The smallest dose that resulted in greater than 90% cure when administered two generation times before death would have occurred, was found to be 10 μg/g body weight. Berenil cure is permanent; no recurrence of infection has been observed in mice examined up to six months after a cure was achieved. The disease is invariably fatal if left untreated, death of the animal occurring in from two to seven days, depending on the initial number of infective trypanosomes administered. No natural immunity or spontaneous cure has been observed in any mice used in the course of this study.

Effect of Berenil Treatment on the Ultrastructure of T. brucei.

Electron microscopic examination of T. brucei harvested at different times after in vivo treatment with a curative dose of Berenil (10 μg/g body weight) reveals a sequence of ultrastructural alterations. Alterations in the KP-DNA are observable soon after Berenil treatment, whereas alterations in the nucleolus are observed only after the elapse of several generation times (generation time = 5-5.5 hrs.) When trypanosomes are examined after four hours of Berenil treatment in vivo, the KP-DNA in nearly all cells appears altered. It seems to have lost its normal filamentous rod-like 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 control cells (Figs. 1, 2 and 6). When trypanosomes are examined several generation times (eighteen hours $\sim$ 3.5 generation times) after Berenil treatment in vivo, pronounced alterations are observed in the nucleus, specifically the nucleolus, of the cells. 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 100% of the cells. (Figs. 7a, 7b and 7c).

MacAdam and Williamson (1972) have examined the effect of in vivo Berenil treatment on bloodstream T. rhodesiense and have found that after six hours of treatment with a curative dose of Berenil, alterations of the KP-DNA (as described above) are evident. No alterations in the nucleus or nucleolus were evident after six hours of Berenil treatment.

If DEAE purified trypanosomes are resuspended in buffer 2 contain-

Key to Symbols used in Electron Micrographs

cr	=	cristae
K	=	kinetoplast
KD	=	kinetoplast DNA
Km	=	kinetoplast membrane
M	=	mitochondrion
Mt	=	subpellicular microtubles
N	=	nucleus
n	=	nucleolus
nf	=	nucleolar fragments
Nm	=	nuclear membrane
P	=	pellicle

Figure 1: Longitudinal section of the 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 untreated 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

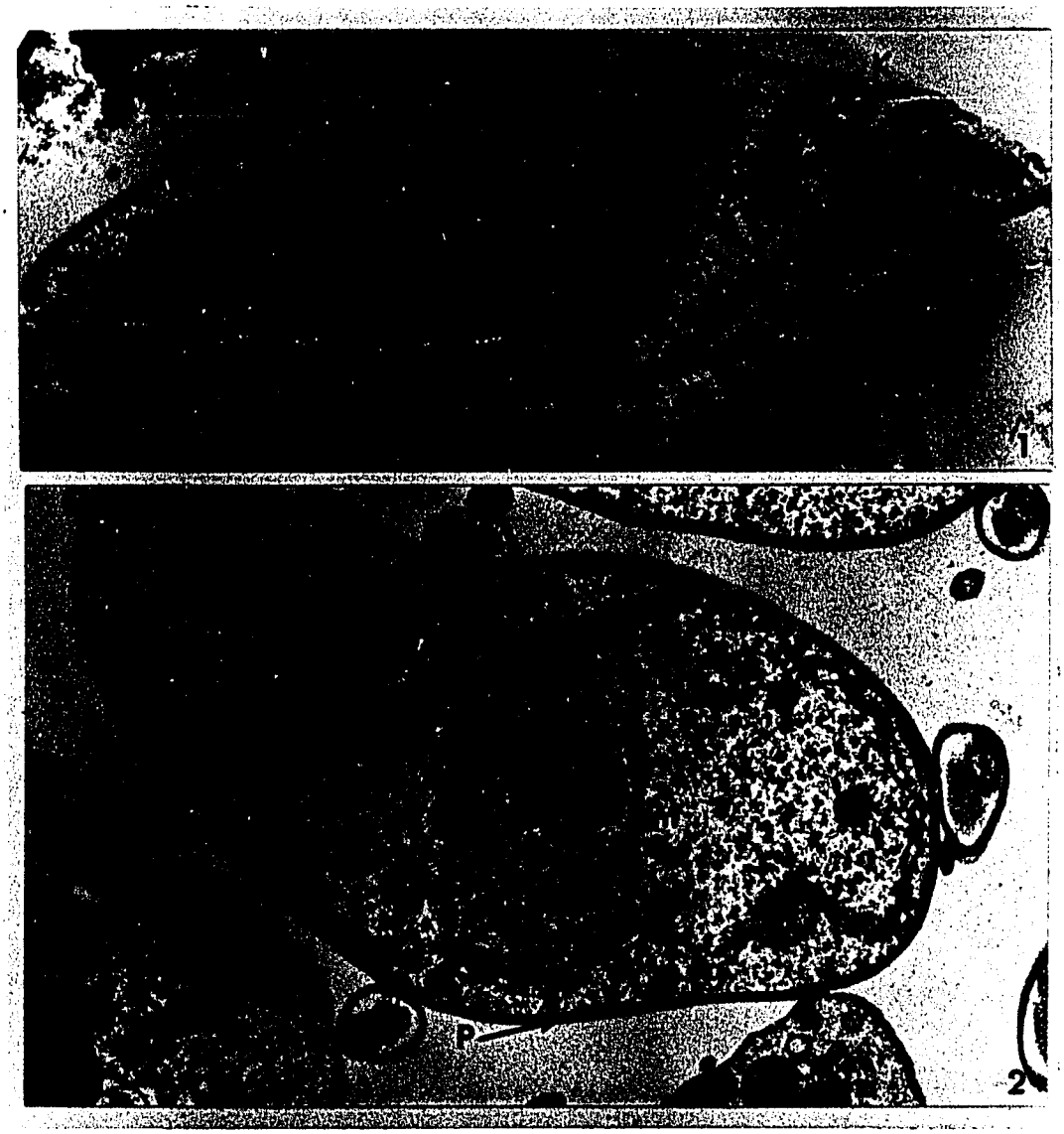
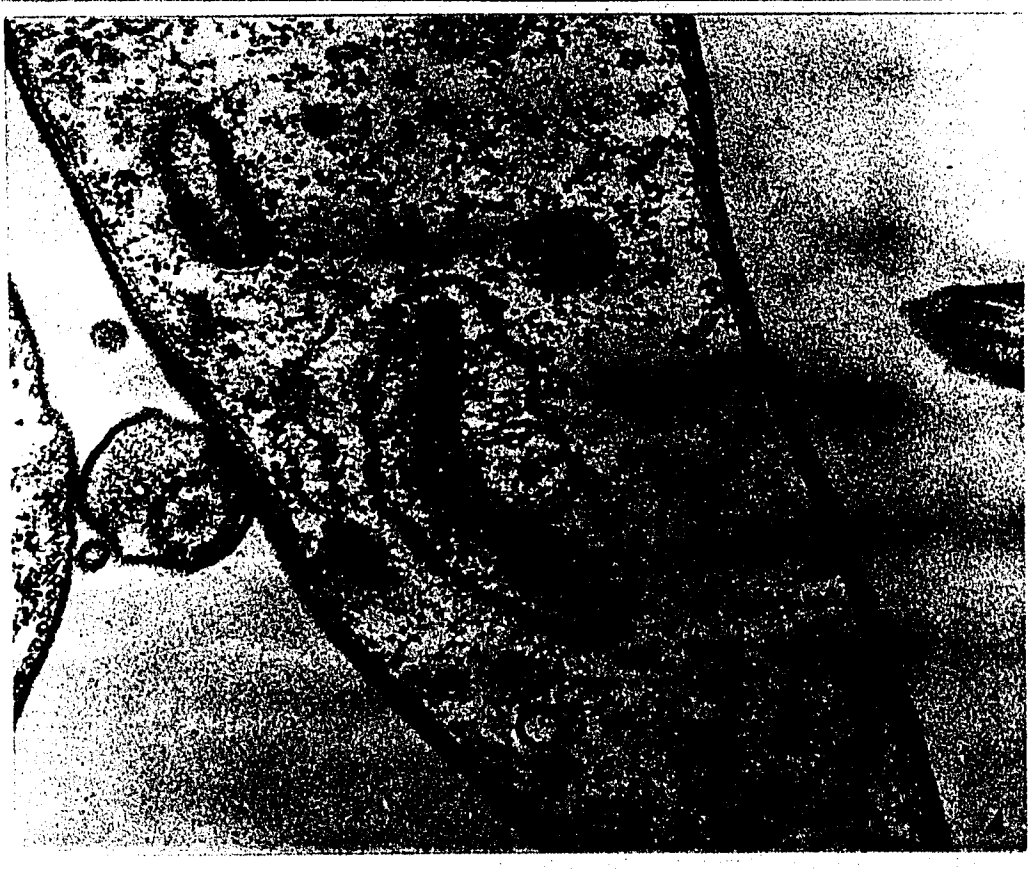

1
P
2

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


3



Figures 5a and 5b: Longitudinal sections of T. brucei exposed in vivo for four hours to a curative dose of Berenil (10 µg/g). 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 dose of Berenil (10 µg/g) showing nucleus and nucleolus. x 17,850

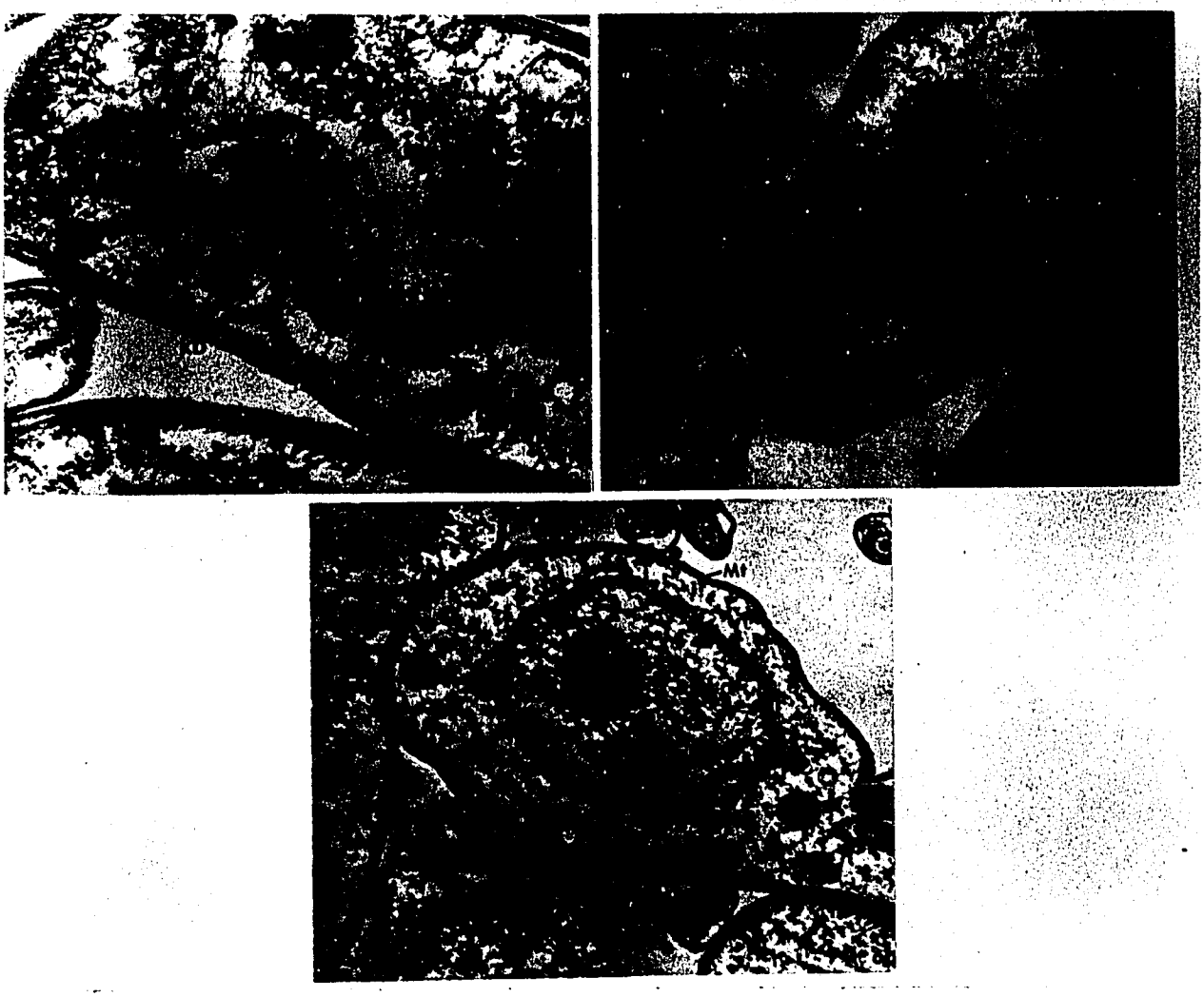
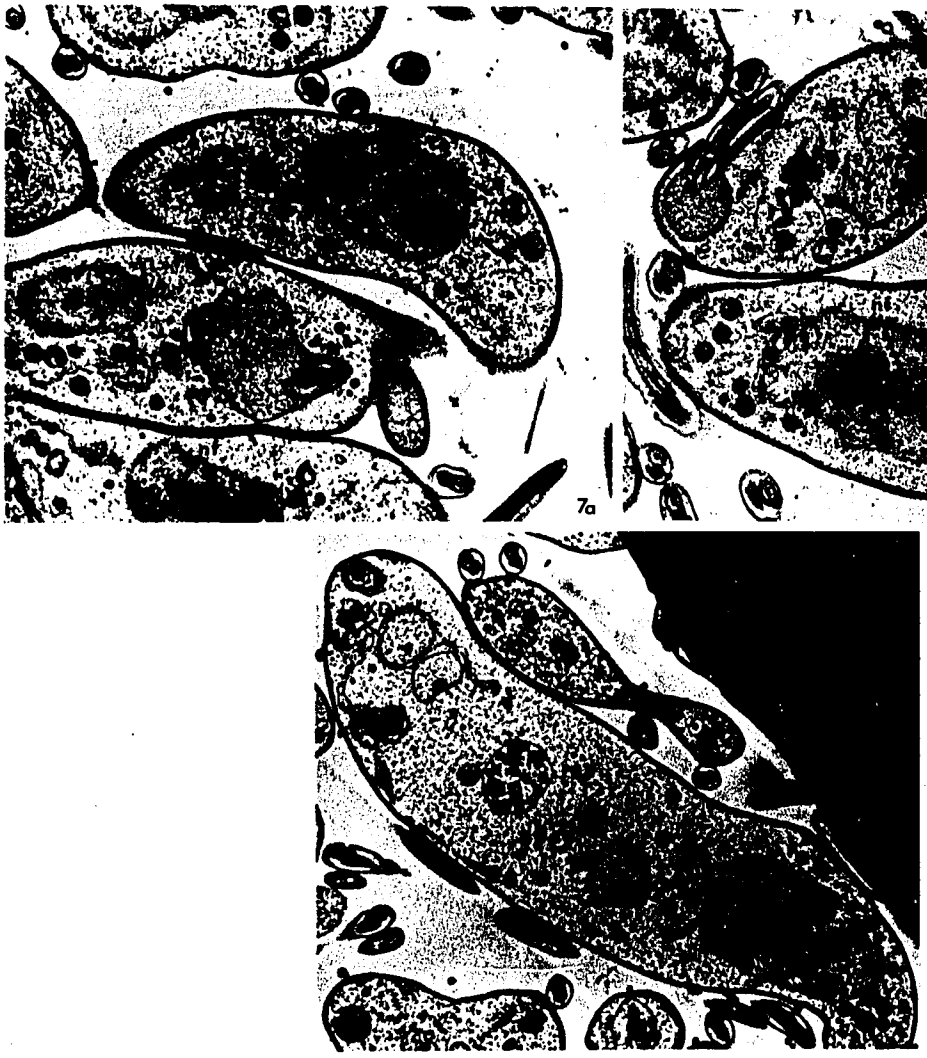

MI

Figure 7a,b,c: Longitudinal sections of T. brucei exposed in vivo for eighteen hours to a curative dose of Berenil (10 µg/g). KP-DNA appears fragmented, as it did in Figs. 5a and 5b. Nucleolar condensation or fragmentation is evident.

a) x 7580

b) x 8660

c) x 6500



ing Berenil at a concentration of 2-10 µg/ml they live for three to four hours when kept at 25°C. When trypanosomes were examined after two hours of exposure to 10 µg/ml Berenil at 25°C, nearly all the cells contained fragmented KP-DNA. The nucleus and nucleolus appeared unaltered. Treatment of trypanosomes under the above in vitro conditions with higher concentrations of Berenil (30-100 µg/ml) caused rapid cell death, followed by lysis.

Although it has been reported that nucleolar fragmentation immediately precedes cell division in T. brucei (Vickerman and Preston, 1970), nucleolar fragmentation was rarely observed in our electron microscopic examination of untreated control trypanosomes. The large number of what appear to be predivisional forms observed after eighteen hours of Berenil treatment in vivo suggested that Berenil-treated cells were unable to divide. It has been shown that Berenil inhibits KP-DNA synthesis in T. mega grown in culture (Newton and Le Page, 1967).

A blockage of cell division may be caused by a breakdown in a number of essential steps which occur during the S and M phases. One of the most basic is inhibition of DNA synthesis. Therefore, the next series of experiments was performed to determine if Berenil inhibits nucleic acid synthesis in trypanosomes resident in the mouse bloodstream.

Effect of Berenil on Incorporation of ^{3}H-Thymidine and ^{3}H-Uracil into the DNA and RNA of Trypanosomes In Vivo.

a) Availability of ^{3}H-Tdr and ^{3}H-U to trypanosomes in the blood of untreated and Berenil-treated animals.

Figures 8 and 9 show the levels of ^{3}H-Tdr and ^{3}H-U respectively in the sera of untreated and Berenil-treated mice at various times after IP

Figure 8: Comparison of serum levels of ^{3}H-thymidine in Berenil-treated and untreated mice infected with T. brucei. At zero time, three mice were injected with either buffer 2 (0.2ml.) or Berenil (10 µg/g) dissolved in buffer 2 (0.2ml.) 30 minutes later, all animals were injected IP 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.

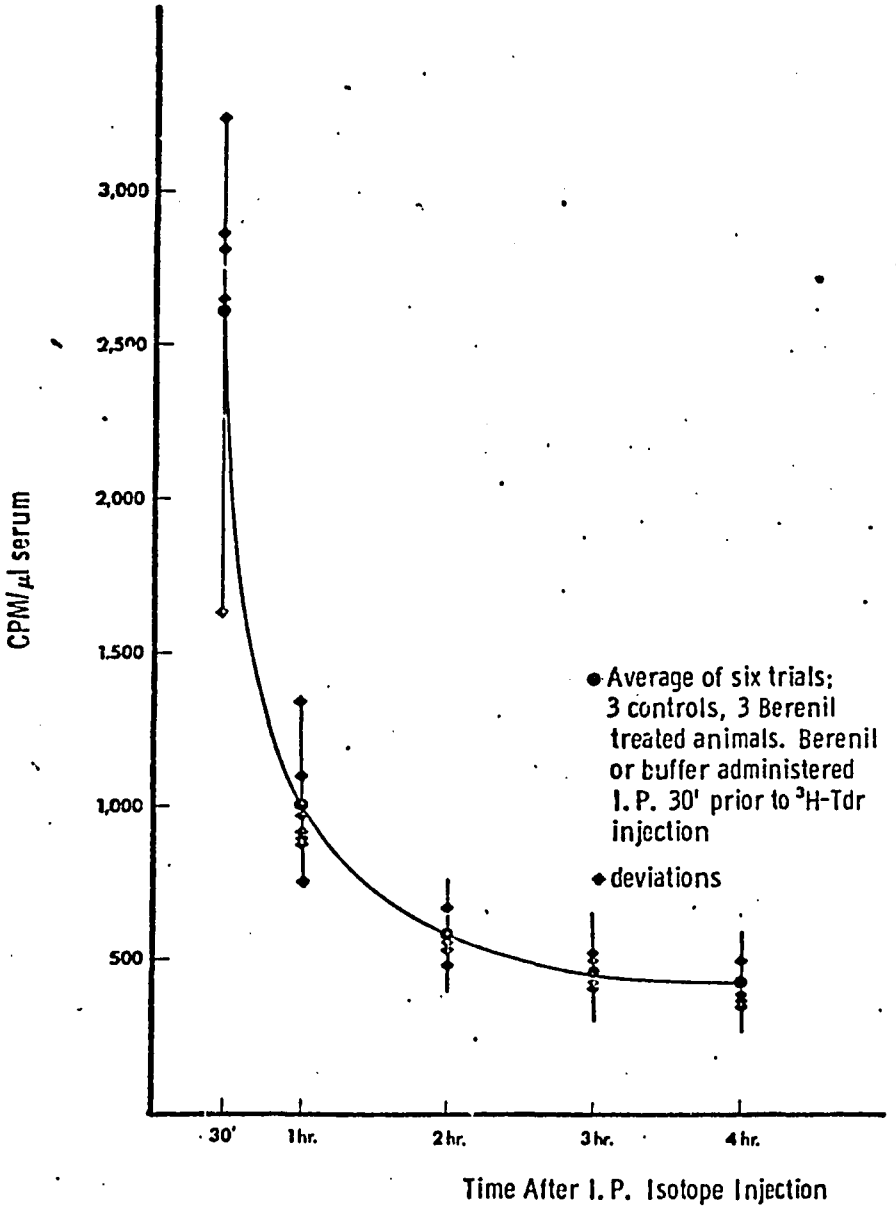
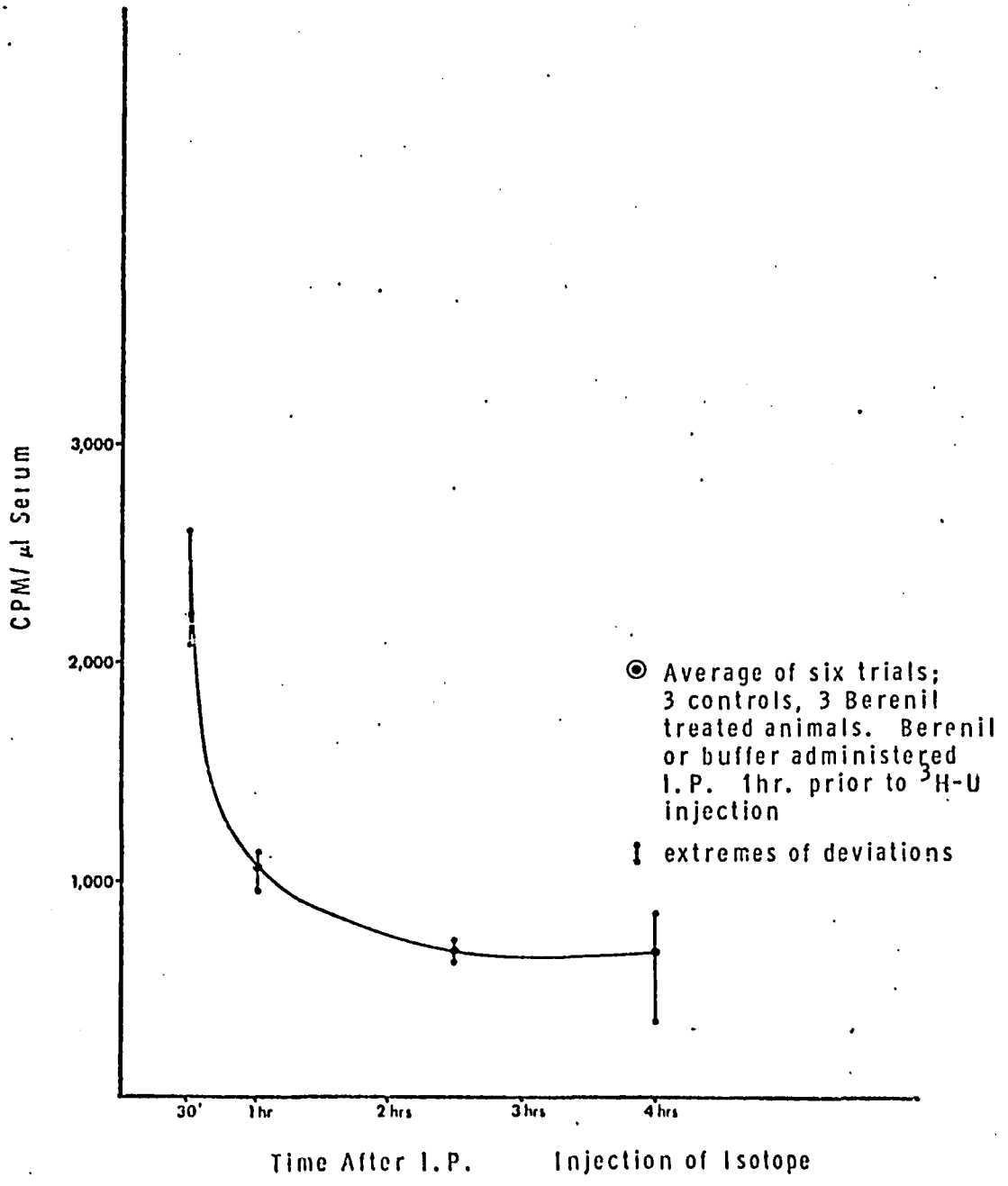

3,000
2,500
2,000
1,500
1,000
500
CPM/μl serum
30'
1hr.
2hr.
3hr.
4hr.
Time After I. P. Isotope Injection
● Average of six trials; 3 controls, 3 Berenil treated animals. Berenil or buffer administered I. P. 30' prior to ³H-Tdr injection
◆ deviations

Figure 9: Comparison of serum levels of ^{3}H-uracil in Berenil-treated and untreated mice infected with T. brucei. Same as Fig. 8 except that Berenil-treated and control animals were injected IP with ^{3}H-uracil (0.5 mCi/0.25ml.) 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.

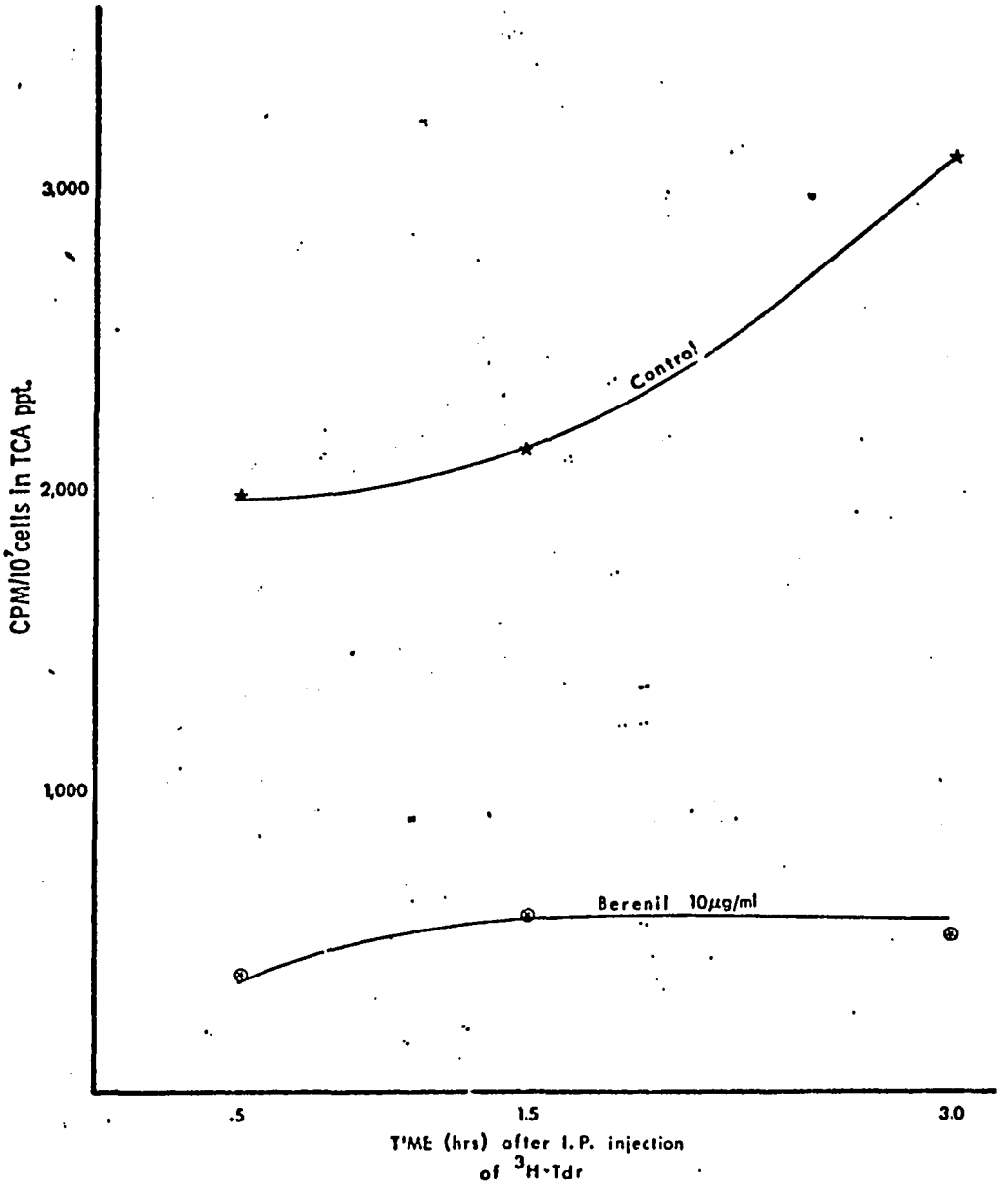

CPM/μl Serum
3,000
2,000
1,000
30'
1 hr
2 hrs
3 hrs
4 hrs
Time After I.P. Injection of Isotope
Average of six trials; 3 controls, 3 Berenil treated animals. Berenil or buffer administered I.P. 1hr. prior to ³H-U injection
extremes of deviations

injection of either ^{3}H-Tdr or ^{3}H-U. It can be seen that Berenil does not interfere with the uptake of ^{3}H-Tdr or ^{3}H-U from the peritoneum nor does its presence in the bloodstream alter the rate at which radioactivity disappears from the bloodstream. Our experiments indicate that this observation is valid provided that injection of ^{3}H-Tdr follows Berenil injection by no less than 30 minutes and injection of ^{3}H-U follows Berenil injection by no less than 60 minutes. Thus, it appears that trypanosomes in untreated and Berenil-treated mice are exposed to near identical levels of radioactive DNA and RNA precursors in the mouse bloodstream.

b) Effect of Berenil on ^{3}H-Tdr incorporation

Figure 10 shows the effect of Berenil (10 μg/g body weight) 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-thymine (see Appendix, Fig. 28). After a 30 minute exposure to Berenil, incorporation of ^{3}H-Tdr into trypanosome DNA is inhibited 70-80%. The labeling of DNA observed in the absence of Berenil is probably a consequence of the continuously decreasing level of isotope in the serum: The greatest incorporation takes place shortly after injection of ^{3}H-Tdr. The accelerating rate of ^{3}H-Tdr incorporation may reflect an intracellular accumulation of ^{3}H-Tdr. During the time course of the experiment described in Figure 10, (0.5-3.0 hours after ^{3}H-Tdr injection) trypanosomes resident in untreated mice

Figure 10: Comparison of ^{3}H-thymidine incorporated into the DNA of *T. brucei* in Berenil-treated and untreated mice. At zero time, control animals were injected with 0.2 ml. buffer 2 and experimental animals received 0.2 ml. Berenil (10 µg/g). 30 minutes later all animals were injected with ^{3}H-thymidine (0.3 mCi/0.3ml.). 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. After RNA hydrolysis the precipitates were filtered and counted by the liquid scintillation method. Each point on the curve represents the average of values obtained from three mice.


3,000
2,000
1,000
CPM/10²cells in TCA ppt.
Control
Berenil 10μg/ml
.5
1.5
3.0
TIME (hrs) after I.P. injection
of ³H-Tdr

incorporate 8-9 times more ^{3}H-Tdr than do trypanosomes resident in Berenil-treated mice.

If the same experiment as that described in Figure 10 is performed except that one-tenth the amount of Berenil (1 μg/g body weight) is administered the inhibition of incorporation of ^{3}H-Tdr into trypanosome DNA is only 25-30%. Thus it appears that the inhibition of incorporation observed is dependent on the amount of Berenil administered.

Like other diamidines, the level of Berenil in the serum of treated animals reaches a maximum quickly and falls rapidly after IP injection of the drug (Hawking, 1963). The experiment described in Figure 11 was performed to determine whether Berenil inhibition of ^{3}H-Tdr incorporation was reversible. It can be seen that when ^{3}H-Tdr is administered 4-5 hours after Berenil treatment (10 μg/g body weight) the inhibition of incorporation is substantially (80% vs 40%) less than the inhibition observed 30 minutes after Berenil treatment. This result suggests that some resumption of ^{3}H-Tdr incorporation occurs several hours after Berenil treatment (probably as a result of removal of Berenil from the mouse bloodstream), and suggests that Berenil inhibition of ^{3}H-Tdr incorporation *in vivo* is reversible.

c) Effect of Berenil on ^{3}H-U incorporation

Figure 12 shows the effect of Berenil (10 μg/g body weight) 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 of trypanosomes labeled *in vivo* with ^{3}H-U. The radioactive material recoverable from 70% PCA hydrolysates of dilute acid insoluble

Figure 11: Relative amount of ^{3}H-thymidine incorporated into trypanosome DNA at various times after treatment with Berenil *in vivo*. At zero time, control animals were injected with buffer 2 (0.2 ml) and experimental animals were injected with Berenil (10 μg/g, 0.2 ml). At 30 minutes, 4 hours, and 5 hours after injection of buffer or Berenil, animals were injected with ^{3}H-thymidine (0.3mCi/0.3 ml). After two hours of exposure to the isotope, trypanosomes were harvested and purified. Triplicate samples 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.

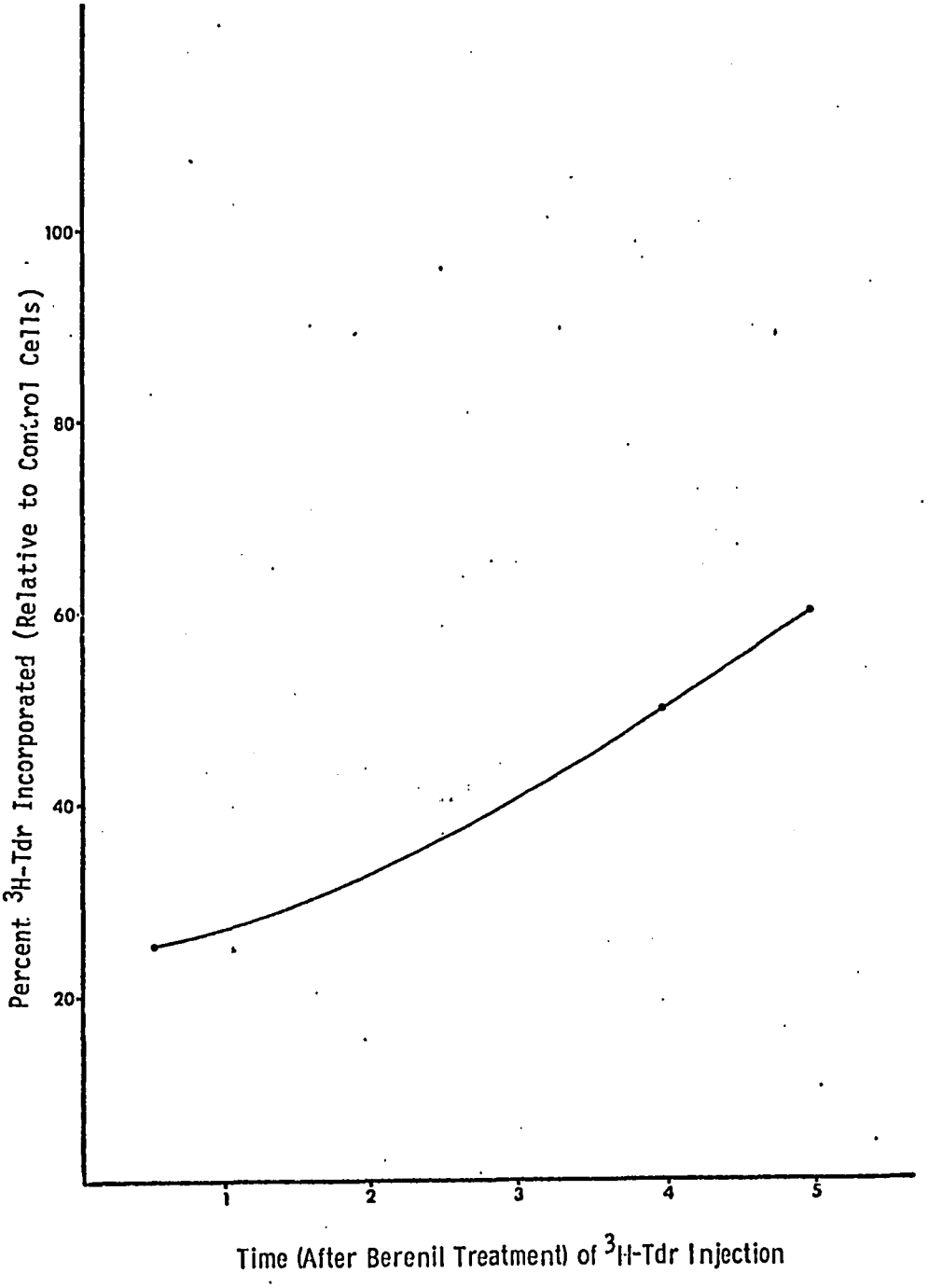
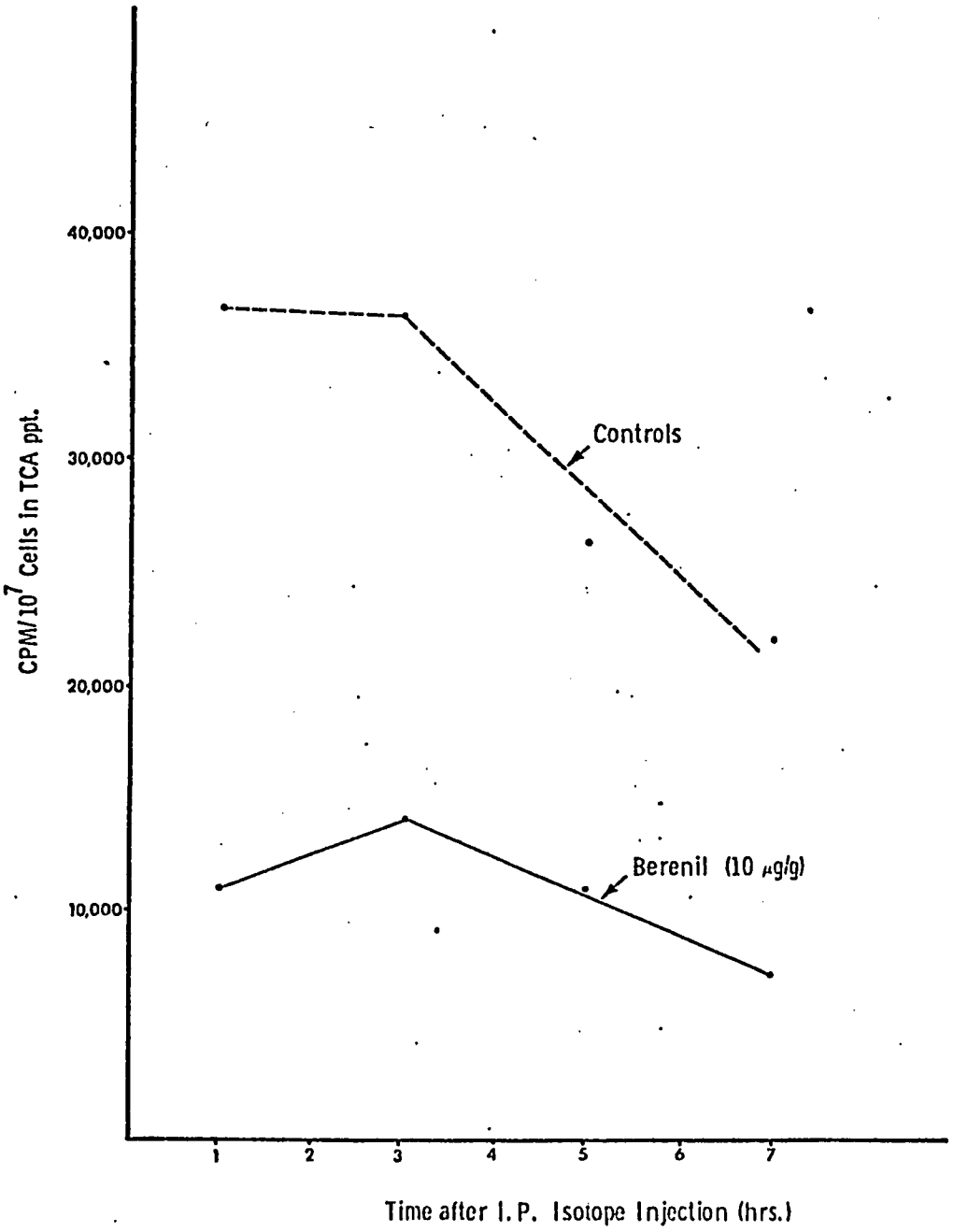

Percent ^{3}H-Tdr Incorporated (Relative to Control Cells)
100
80
60
40
20
1
2
3
4
5
Time (After Berenil Treatment) of ^{3}H-Tdr Injection

Figure 12: Comparison of ^{3}H-uracil incorporated into RNA of T. brucei in Berenil-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 Berenil or buffer. Blood was harvested from control and experimental animals 1, 3, 5, and 7 hours after injection with ^{3}H-uracil.


40,000
30,000
20,000
10,000
CPM/10^7 Cells in TCA ppt.
Controls
Berenil (10 μg/g)
1
2
3
4
5
6
7
Time after I.P. Isotope Injection (hrs.)

extracts of trypanosomes labeled with ^{3}H-U was almost exclusively uracil and cytosine. Only a small amount of radioactive thymine (<5%) was detected (see Appendix, Fig. 29). The sum of the synthesis and degradation of RNA occurring in the presence of a continously decreasing concentration 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 hours after administration of ^{3}H-U.

An inference made from Figure 12, is that less ^{3}H-U (about 60% less) is incorporated into rapidly labeled RNA in trypanosomes present in Berenil-treated vs. untreated mice.

If the same experiment as that described in Figure 12 is performed except that one-tenth the amount of Berenil (1 μg/g body weight) is administered the inhibition of incorporation of ^{3}H-U into rapidly labeled trypanosome RNA is only 20%. Thus it appears that the inhibition of incorporation observed is dependent on the amount of Berenil administered.

In order to determine if Berenil inhibition of ^{3}H-U incorporation is reversible, the isotope was administered 1 hour and 3 hours after Berenil treatment. When ^{3}H-U is administered 1 hour after Berenil treatment (10 μg/g body weight) the inhibition of incorporation is 60% as opposed to a 30% inhibition of incorporation after 3 hours of Berenil treatment. This result suggests that some resumption of ^{3}H-U incorporation occurs several hours after Berenil treatment (probably as a result of removal of Berenil from the mouse bloodstream), and suggests that Berenil inhibition of ^{3}H-U incorporation *in vivo* is reversible.

Effect of Berenil on the Ratio of DNA/RNA/Protein in T. brucei In Vivo.

Table II 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 curative dose of Berenil (10 μg/g body weight). The DNA content was determined by the p-nitrophenylhydrazione method, (Webb and Levy, 1955). The RNA content was determined by the orcinol method (Schneider, 1937). The RNA values were corrected for errors caused by the presence of DNA. The protein content was determined by the Lowry method (Lowry, et al., 1951). The results shown in Table II are consistent with those reported by Riou and Pautrizel (1969) who have determined the DNA and RNA contents of several species of trypanosomes. DNA values range from 0.77-1.73 x 10^{-7} (μg/trypanosome) and RNA values range from 5-30 x 10^{-7} (μg/trypanosome).

The data in Table II suggest that Berenil-treated trypanosomes are able to double their DNA, RNA and protein content, but are unable to divide.

Effect of Berenil on Incorporation of ^{3}H-Thymidine, ^{14}C-Uracil and ^{14}C-Leucine into DNA, RNA and Protein In Vitro.

At present, no medium is available which supports in vitro cultivation of bloodstream trypanosomes. Monomorphic bloodstream trypanosomes when placed in culture media, can be maintained for varying periods of time (depending on conditions of temperature and pH) but do not divide and eventually die (Vickerman, 1971). Numerous experiments can be performed on cells in culture (i.e. pulse and chase experiments) that would be impossible to perform on trypanosomes in the mammalian bloodstream. In the hopes of extending our knowledge of the mode of action of Berenil, we have

LEGEND: TABLE II

DNA, RNA and protein content of untreated and Berenil-treated trypanosomes were determined as follows: Twelve mice with high parasitemia levels (100-150 trypanosomes/40xfield) were used as a source of trypanosomes. The mice were divided into two groups: Trypanosomes were harvested from six of the mice (untreated trypanosomes) and the remaining six mice were injected (IP) with Berenil (10 μg/g). Twelve hours later, trypanosomes were harvested from the Berenil treated animals (trypanosomes from Berenil treated mice). After being passed through DEAE cellulose, trypanosomes (from both untreated and Berenil-treated mice) were counted, divided into 2 aliquots, washed with Buffer 2 and resuspended in a known concentration in either 5% TCA, or Buffer 2. Trypanosomes resuspended in 5% TCA were hydrolyzed for 30 minutes at 100°C, and those resuspended in Buffer 2 were homogenized. DNA and RNA determinations were carried out using 5% TCA hydrolysates and protein determinations were carried out using the homogenate. The experiment outlined above was performed twice and all DNA, RNA and protein determinations were performed in triplicate. The values presented in Table II are average values. The deviations from these values are as follows: DNA ± 10%, RNA ± 15%, protein ± 5%.

TABLE II

Effect of Berenil on DNA, RNA and Protein Content of *T. brucei*

	DNA(μg/cell)	RNA(μg/cell)	Protein(μg/cell)
Untreated Trypanosomes	1.7×10^{-7}	8.6×10^{-7}	60.7×10^{-7}
Trypanosomes from Berenil-treated mice (10μg/g; 12 hrs.∿2.2 generation times)	3.0×10^{-7}	20.4×10^{-7}	120.9×10^{-7}

attempted to determine some *in vitro* correlates of the effects of Berenil observed *in vivo*. To accomplish this, we have performed some "washed cell" experiments in which DEAE-purified trypanosomes were suspended in buffer 2. We attempted to determine whether trypanosomes are able to synthesize nucleic acids and proteins under these conditions. Buffer 2 is a strongly buffered suspending medium, in which glucose is present as an energy source. Trypanosomes suspended in this medium are motile for 3-4 hours at 37°C (at 3.5 hours, 50% of the cells are non-motile) and 5-6 hours at 25°C (at 6 hours, 50% of the cells are non-motile). Trypanosomes in buffer 2 are therefore dying cells, and the incorporation of nucleic acid and protein precursors observed under these conditions reflects a minimal (endogenous) synthesis. We tried suspending trypanosomes in Dulbecco's medium (+ fetal calf serum) in the hopes of prolonging viability *in vitro*, but the cells died at the same time in Dulbecco's medium as they did in buffer 2.

That the trypanosomes are metabolically active under these *in vitro* conditions can be seen in Figure 13, which reveals that trypanosomes excrete a large amount of pyruvate into the suspending medium during *in vitro* incubation. The accumulation of pyruvate in the suspending medium is linear with time from 0 to 3.5 hours. These *in vitro* studies were not performed under sterile conditions. The incorporation of ^{3}H-Tdr into DNA and ^{14}C-Leu into protein under *in vitro* conditions were unaffected by penicillin (100 U/ml) and streptomycin (100 µg/ml), and the amount of labeled precursor incorporated was directly proportional to the concentration of trypanosomes in the suspending medium. This observation suggests that we were measuring radioactive precursor incorporation by trypanosomes, and not that by bacterial contaminants.

Figure 14 indicates that only a small amount of ^{3}H-Tdr is incorporated into trypanosome DNA (defined as in the *in vivo* experiments - cold

Figure 13: Spectra of suspending buffer and sodium pyruvate. DEAE-purified trypanosomes were suspended in buffer 2 for 3.5 hours at 37°C at a concentration of 10^7 cells/ml. After removing the cells by centrifugation, the absorption spectrum of the suspending buffer was determined. The absorption spectrum of sodium pyruvate is shown for reference.

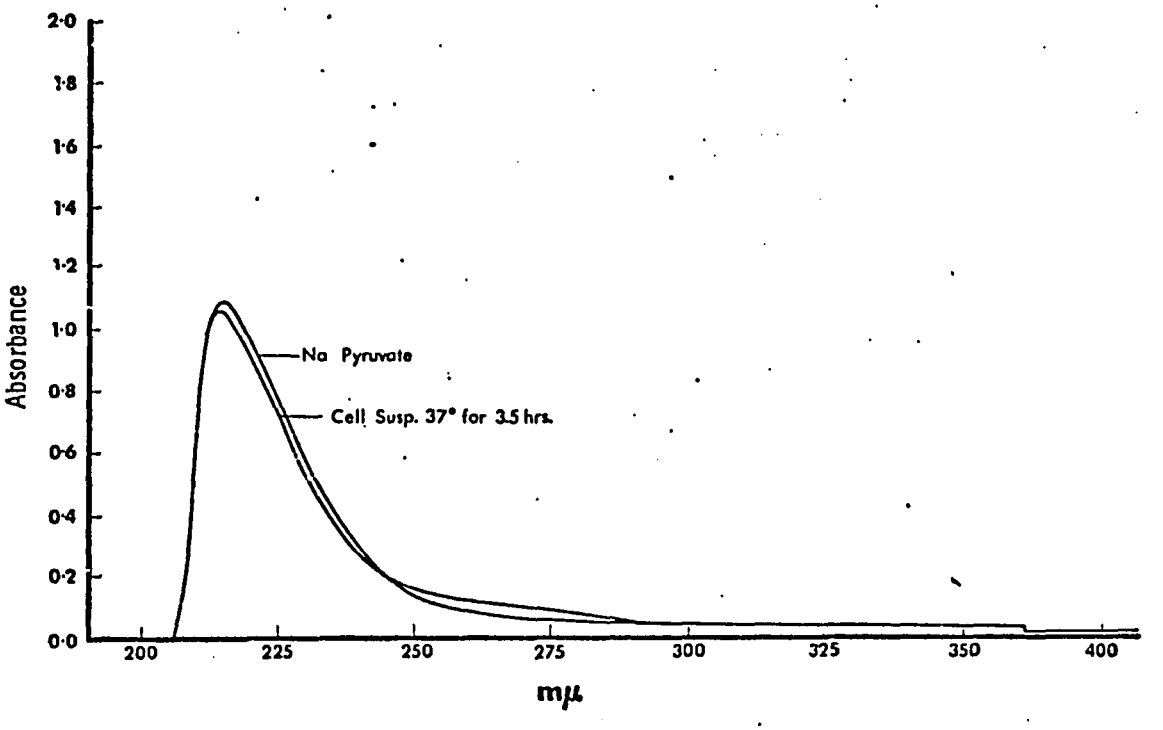
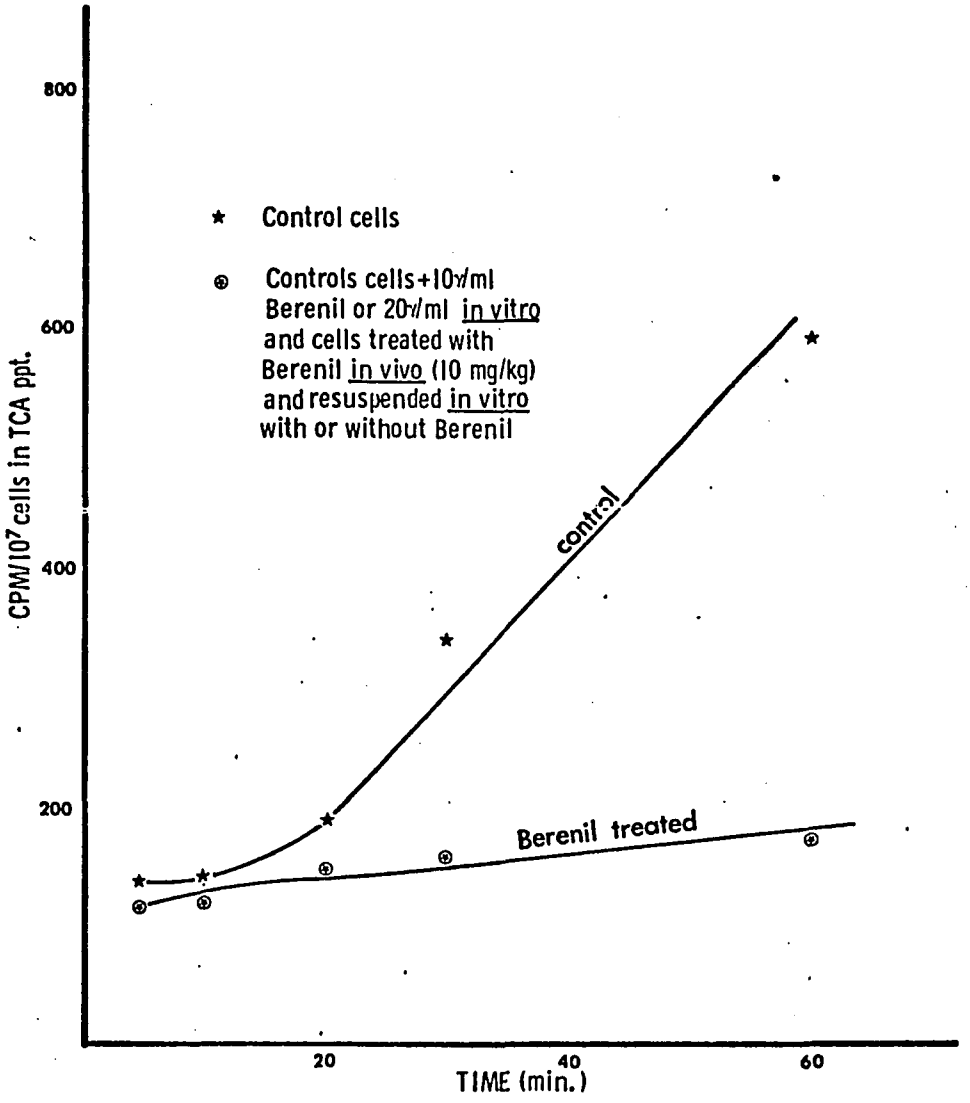

2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0·0
Absorbance
Na Pyruvate
Cell Susp. 37° for 3.5 hrs.
200
225
250
275
300
325
350
400
mμ

Figure 14: Effect of Berenil on incorporation of ^{3}H-Tdr into DNA at 25°C *in vitro*. DEAE-purified trypanosomes isolated from untreated mice (controls) and Berenil-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 Berenil and with Berenil (at 10 μg/ml and 20 μg/ml) and twenty-five ml. aliquots of trypanosomes from Berenil-treated mice were incubated without Berenil and with Berenil (10 μg/ml). Berenil 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.


800
600
400
200
CPM/10⁷ cells in TCA ppt.
★ Control cells
⊛ Controls cells+10γ/ml Berenil or 20γ/ml in vitro and cells treated with Berenil in vivo (10 mg/kg) and resuspended in vitro with or without Berenil
control
Berenil treated
20
40
60
TIME (min.)

5% TCA precipitable, alkali stable material) at 25°C in buffer 2, but that the incorporation which does occur is inhibited by Berenil. Figure 15 shows that appreciable incorporation of ^{3}H-Tdr into trypanosome DNA occurs at 37°C in buffer 2, and that this incorporation is almost completely inhibited by Berenil present at a concentration of 5 μg/ml. Figure 16 suggests that Berenil inhibition of ^{3}H-Tdr incorporation is reversible *in vitro*, i.e. 30 minutes after complete cessation of ^{3}H-Tdr incorporation, cells that are washed and resuspended without Berenil are able to resume ^{3}H-Tdr incorporation. These *in vitro* results are in agreement with the *in vivo* results reported in the previous section: Berenil reversibly inhibits ^{3}H-Tdr incorporation into trypanosome DNA.

Figure 17 reveals that appreciable incorporation of ^{14}C-U into trypanosome RNA (defined as in *in vivo* experiments - cold 5% TCA precipitable, alkali unstable material) occurs at 25°C *in vitro*. In the presence of Berenil (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 Berenil *in vivo* for 30 minutes, prior to being harvested, exhibit immediate inhibition of incorporation whether or not Berenil is added to the incubation medium. However, cells resuspended in the absence of Berenil (plus Ber *in vivo* but minus Ber *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 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 Berenil.

Figures 19a-d show the results of a series of pulse and chase ex-

Figure 15: Effect of Berenil 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 Berenil and in the presence of Berenil (2 μg/ml and 5 μg/ml). Berenil 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.

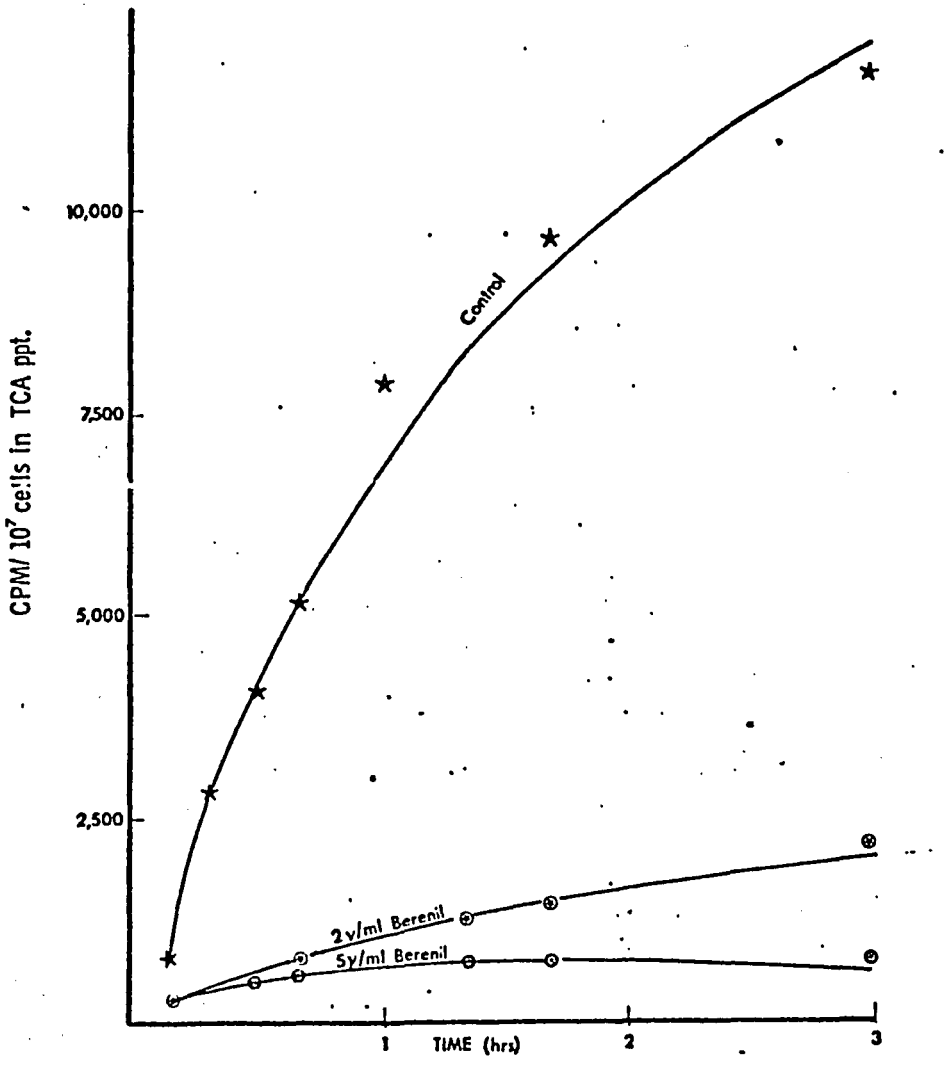

CPM/ 10⁷ cells in TCA ppt.
10,000
7,500
5,000
2,500
Control
2γ/ml Berenil
5γ/ml Berenil
1
TIME (hrs)
2
3

Figure 16: Reversibility of Berenil 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 Berenil. The trypanosomes were removed from the suspending medium by centrifugation and resuspended in buffer 2 without Berenil containing ^{3}H-thymidine (4 µCi/ml). Cell samples were removed and counted as in Fig. 14.

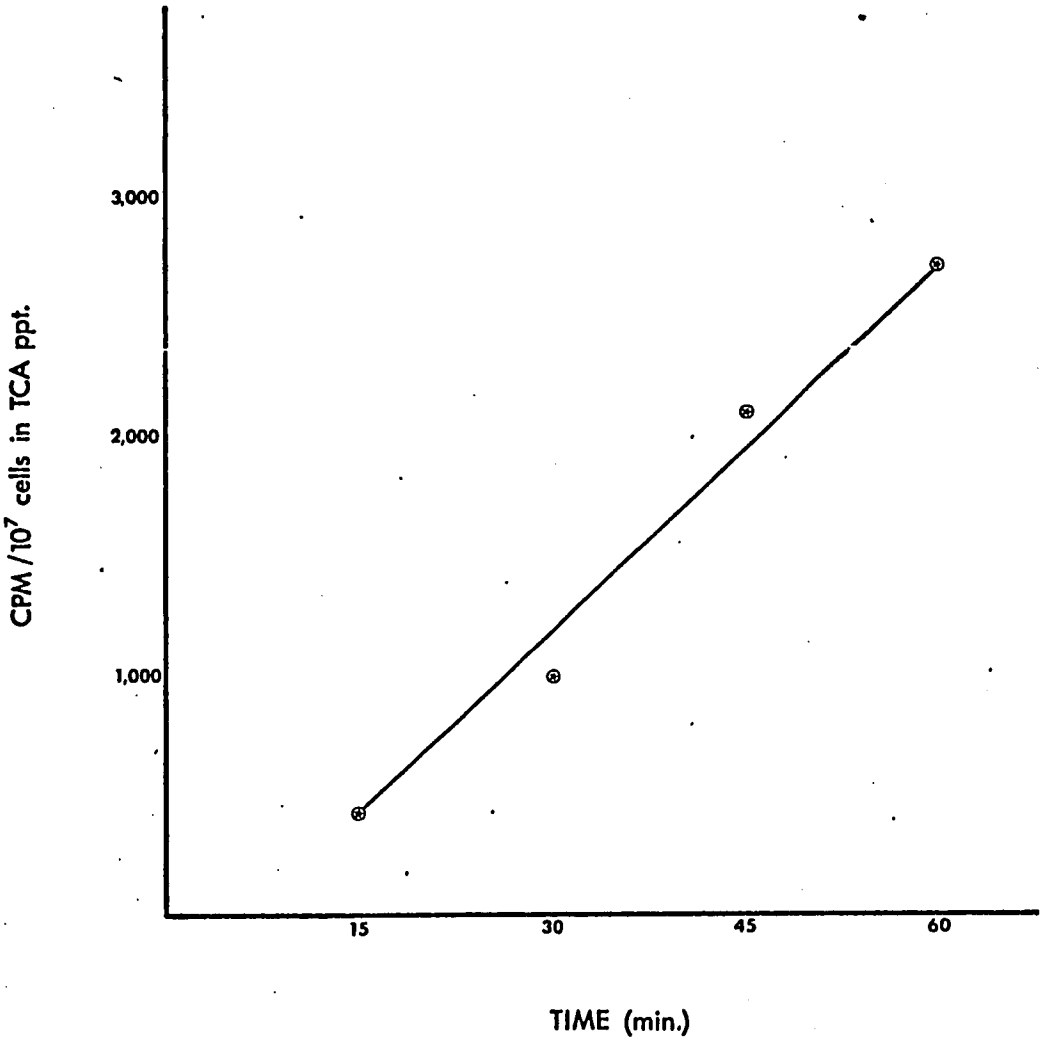

3,000
2,000
1,000
CPM /10^7 cells in TCA ppt.
15
30
45
60
TIME (min.)

Figure 17: Effect of Berenil 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.). Berenil was added at zero time.

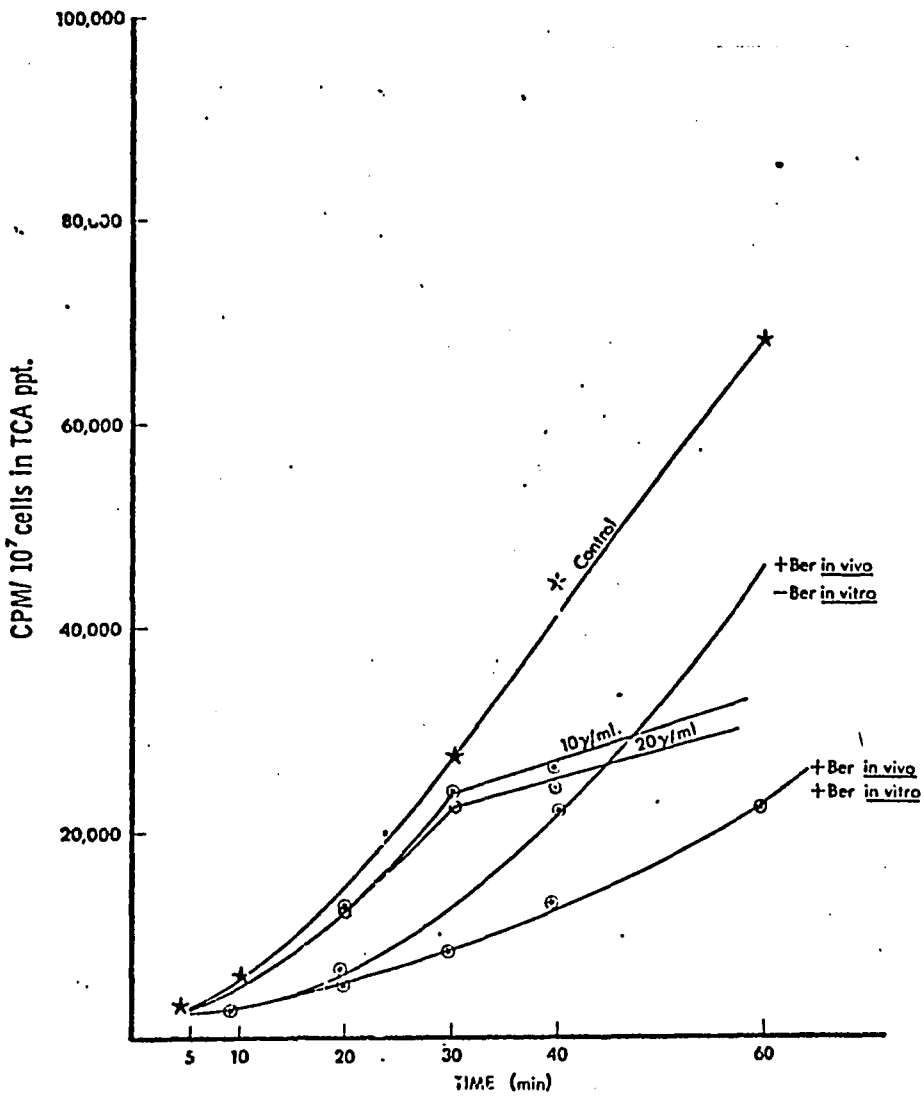
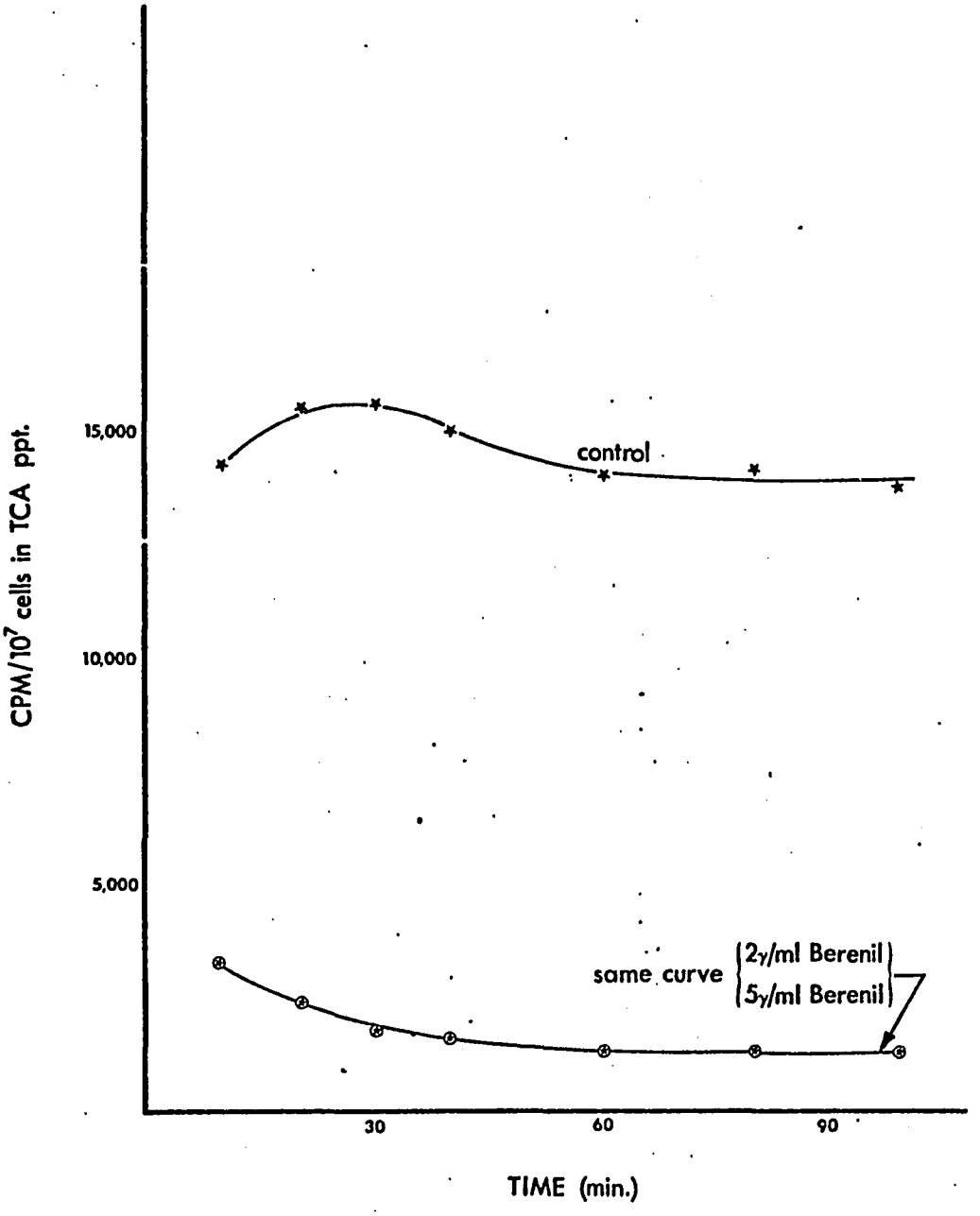

100,000
80,000
60,000
40,000
20,000
CPM/ 10⁷ cells in TCA ppt.
Control
+Ber in vivo
−Ber in vitro
10γ/ml.
20γ/ml
+Ber in vivo
+Ber in vitro
5
10
20
30
40
60
TIME (min)

Figure 18: Effect of Berenil on incorporation of ^{14}C-uracil into RNA at 37°C *in vitro*. Same as Fig. 15, except that trypanosomes were labeled with ^{14}C-uracil (0.6μCi/ml.).


CPM/10⁷ cells in TCA ppt.
15,000
10,000
5,000
control
same curve
2γ/ml Berenil
5γ/ml Berenil
30
60
90
TIME (min.)

periments with ^{14}C-uracil. These results suggest that:

(1) RNA synthesized in the absence of Berenil is degraded more quickly in the presence of Berenil than in the absence of Berenil. That is, pre-existing RNA is degraded more rapidly in the presence of Berenil than in the absence of Berenil.

(2) RNA synthesized in the presence of Berenil is not degraded as rapidly as pre-existing RNA when Berenil is present. That is, RNA synthesized during exposure to Berenil is more stable to degradation than RNA synthesized in the absence of Berenil.

Some hypotheses to account for these observations are presented in Table III.

Figures 20 and 21 reveal the effect of Berenil on incorporation of ^{14}C-leucine into cold 5% TCA precipitable, hot 5% TCA stable material (protein) at 25°C and 37°C in vitro. At both temperatures, Berenil stimulates the incorporation of ^{14}C-Leu into protein. A number of hypotheses may be advanced to account for this observation. The stimulation of incorporation may be a reflection of what happens to RNA in the presence of Berenil (i.e. see Table III, Observation 2, Interpretation 3), or alternatively, it may be a reflection of a physical change in the protein synthesizing machinery of the cell, i.e. Berenil binding to ribosomes might alter the conformation of the ribosomes resulting in an increase in the rate at which they move along the m-RNA.

The results obtained from these in vitro studies suggested that Berenil was able to interfere with DNA and RNA synthesis in trypanosomes. In subsequent experiments, the effect of Berenil on in vitro DNA and RNA synthesizing systems was examined in the hope of determining the mechanism of action of Berenil.

Figure 19a: ^{14}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 cells/ml and warmed to 25°C. Twenty-five ml. aliquots of cells were used. The pulse (^{14}C-uracil, .66μCi/ml) was given in the presence and absence of Berenil (3.3μg/ml), and the chase was performed (by addition of a 1000 fold excess of unlabeled uracil) in the presence and absence of Berenil (3.3μg/ml). The pulse and chase were performed in the following manner:

(1) 5' Pulse (minus Berenil)-chase (minus Berenil)

(2) 5' Pulse (minus Berenil)-chase (plus Berenil)

(3) 5' Pulse (plus Berenil)-chase (plus Berenil)

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.

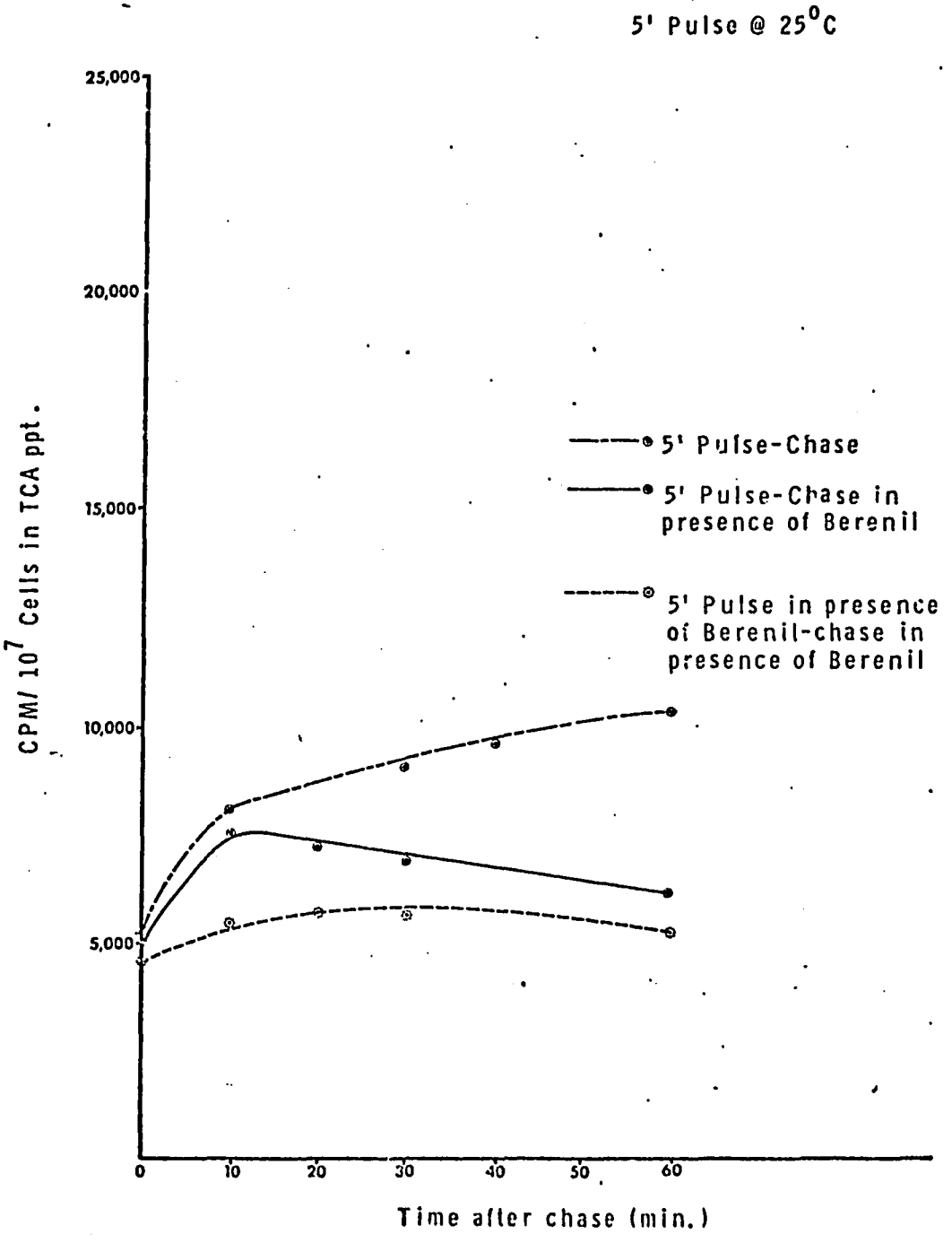

5' Pulse @ 25°C
25,000
20,000
15,000
10,000
5,000
CPM/ 10^7 Cells in TCA ppt.
5' Pulse-Chase
5' Pulse-Chase in presence of Berenil
5' Pulse in presence of Berenil-chase in presence of Berenil
0
10
20
30
40
50
60
Time after chase (min.)

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

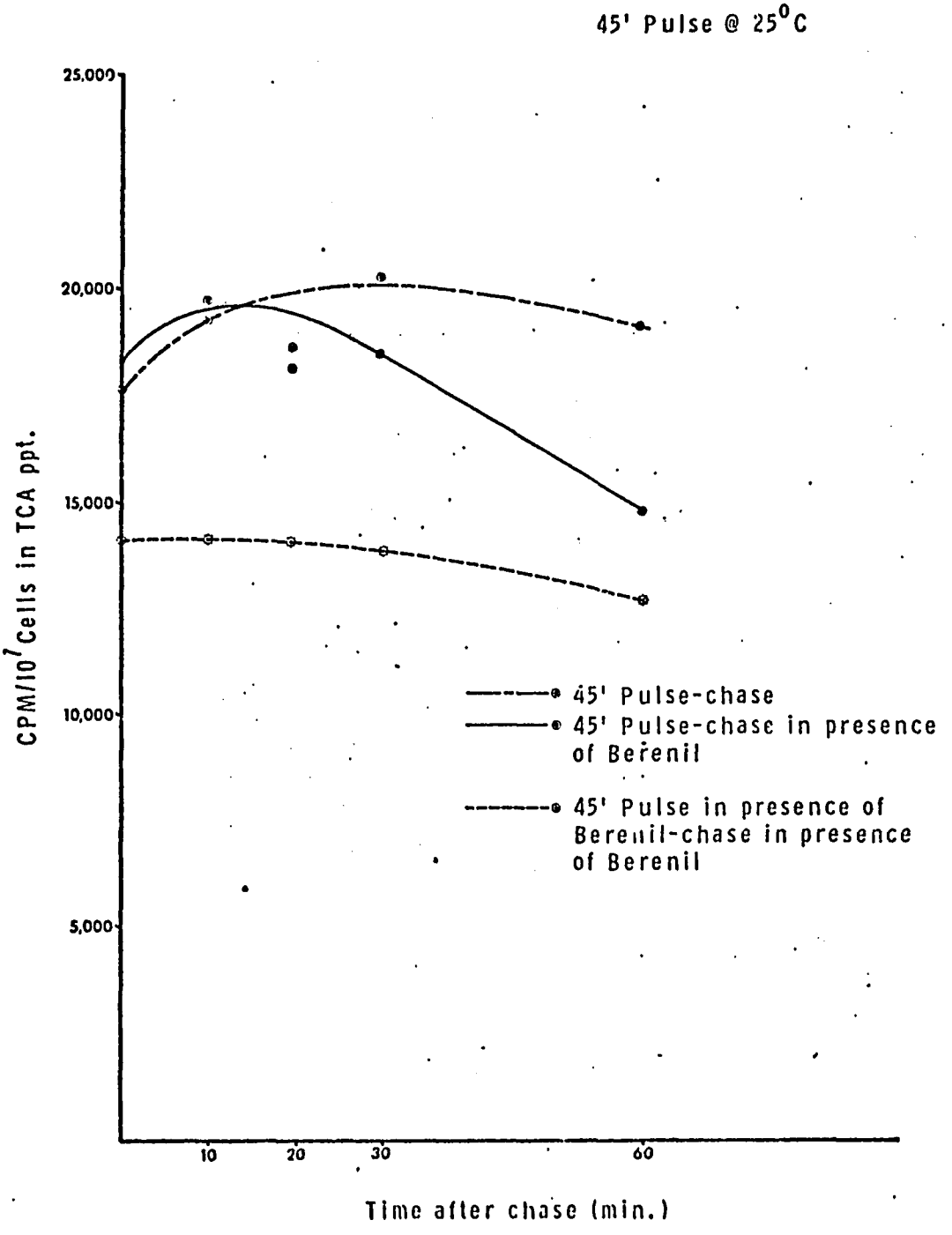

45' Pulse @ 25°C
25,000
20,000
15,000
10,000
5,000
CPM/10^7 Cells in TCA ppt.
45' Pulse-chase
45' Pulse-chase in presence of Berenil
45' Pulse in presence of Berenil-chase in presence of Berenil
10
20
30
60
Time after chase (min.)

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

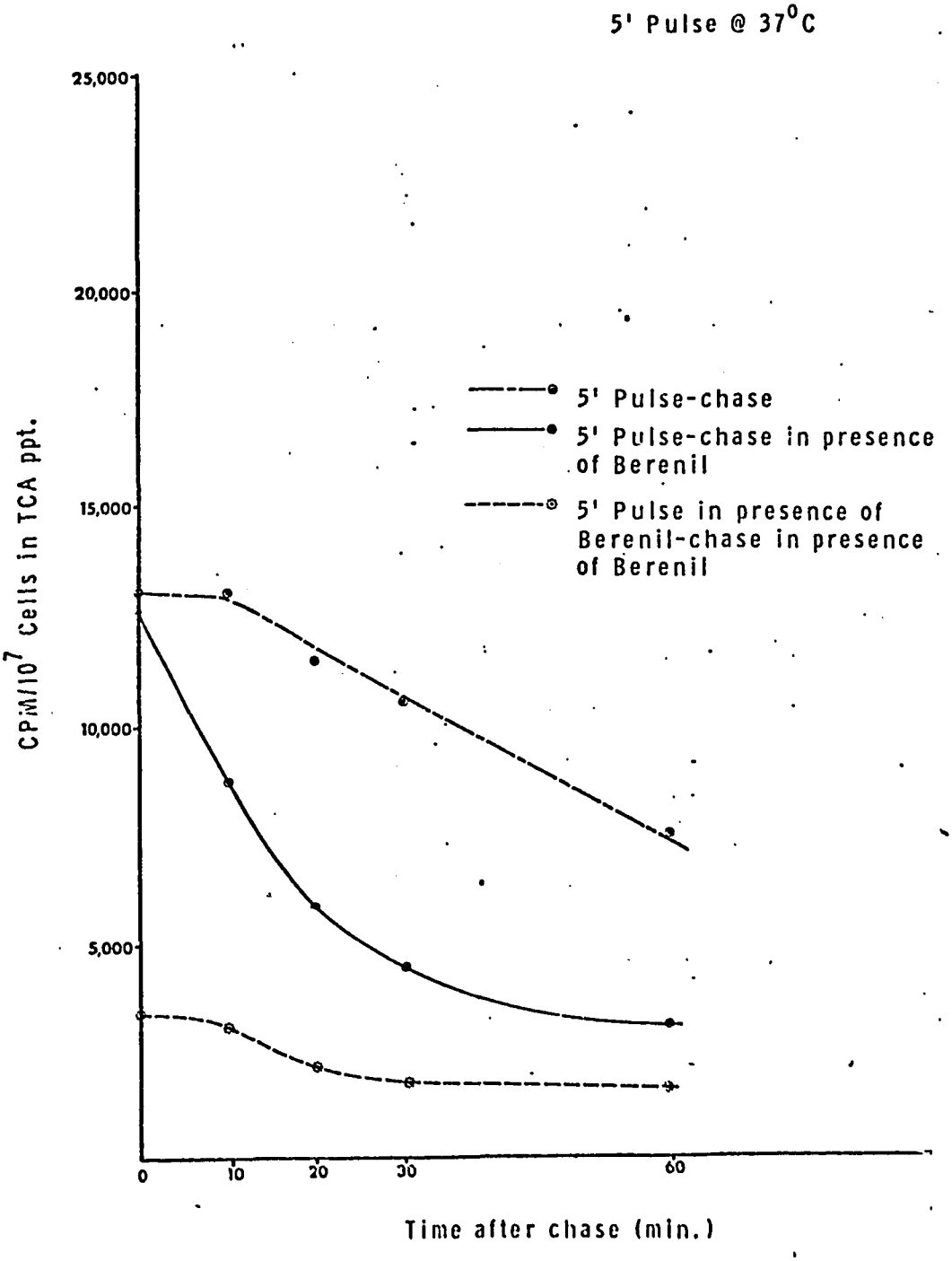

5' Pulse @ 37°C
25,000
20,000
15,000
10,000
5,000
CPM/10^7 Cells in TCA ppt.
5' Pulse-chase
5' Pulse-chase in presence of Berenil
5' Pulse in presence of Berenil-chase in presence of Berenil
0
10
20
30
60
Time after chase (min.)

Figure 19d: ^{14}C-uracil pulse and chase, 45 minute pulse, 37°C. Same as Fig. 19a, except that pulse time was 45 minutes at 37°C.

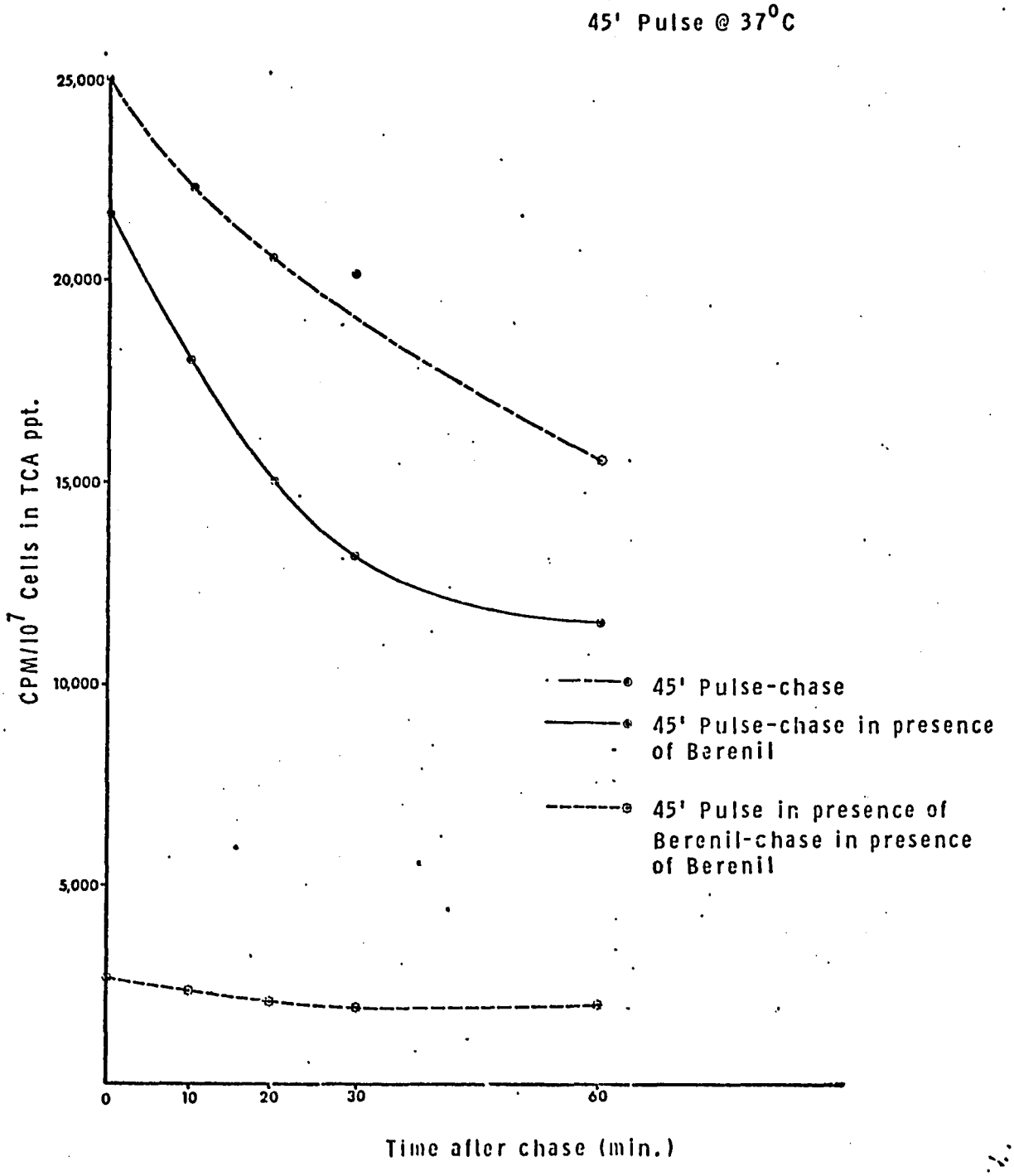

45' Pulse @ 37°C
25,000
20,000
15,000
10,000
5,000
CPM/10^7 Cells in TCA ppt.
0
10
20
30
60
Time after chase (min.)
45' Pulse-chase
45' Pulse-chase in presence of Berenil
45' Pulse in presence of Berenil-chase in presence of Berenil

TABLE III

Some Hypotheses to Account for Data Obtained from ^{14}C-Uracil Pulse and Chase Experiments

Observation	Some Possible Interpretations
(1) Pre-existing RNA is degraded more quickly in the presence of Berenil than in the absence of Berenil.	(1) Berenil activates an RNAase. (2) Binding of Berenil to previously synthesized RNA increases the affinity of the RNA for RNAase. (3) Binding of Berenil to RNA prevents a stabilization which normally occurs, as (a) inhibition of attachment of ribosomes to recently synthesized m-RNA. (b) inhibition of attachment of ribosomal proteins to recently synthesized r-RNA.
(2) RNA made in the presence of Berenil is more stable to degradation than is pre-existing RNA.	(1) RNA made in the presence of Berenil is qualitatively different, i.e. proportionately more stable RNA is made in the presence of Berenil. (2) RNA made in the presence of Berenil is chemically altered and more resistant to RNAase. (3) Altered m-RNA made in the presence of Berenil has a higher affinity for ribosomes than normal m-RNA. (4) RNA made in the presence of Berenil remains attached to the DNA template (This would also account for the 30 minute lag period before inhibition of ^{14}C-U incorporation is observed (Fig. 17) - RNA synthesis stops when all transcription sites are saturated).

Figure 20: Effect of Berenil 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 Berenil (10 μg/ml and 20 μg/ml).

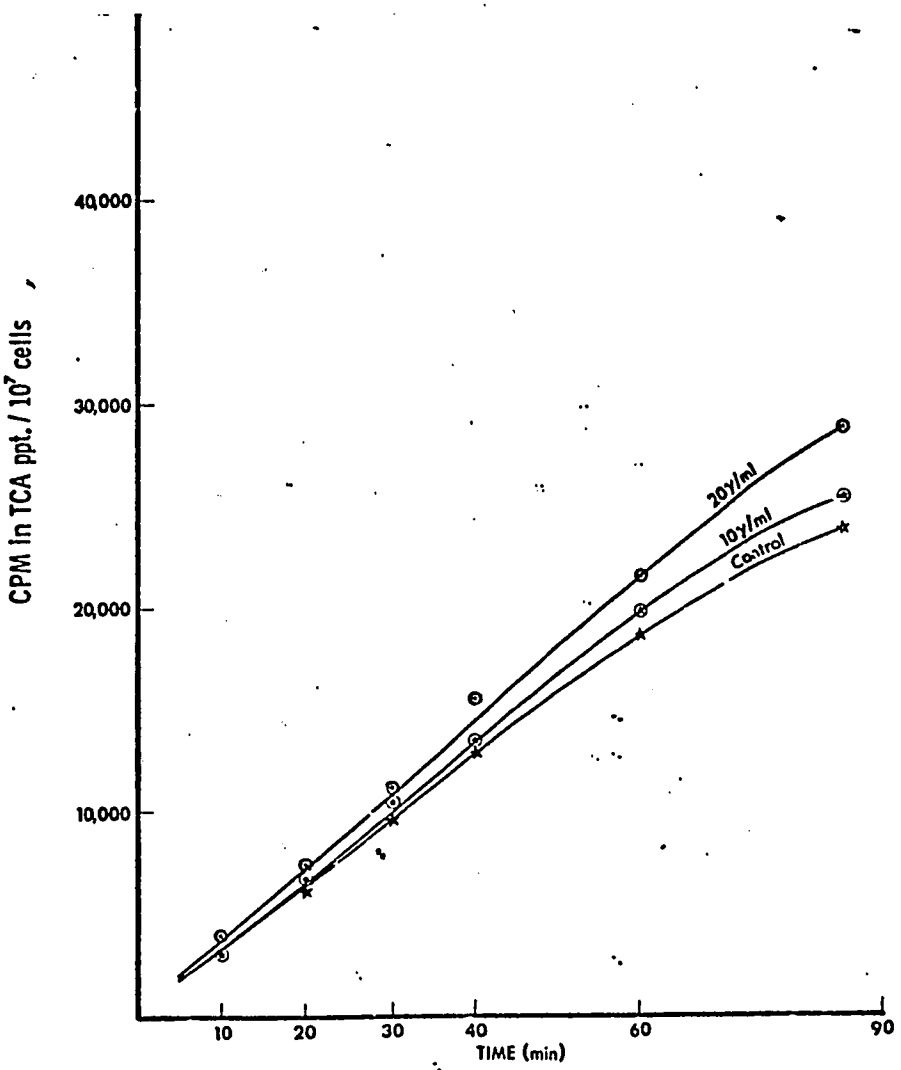
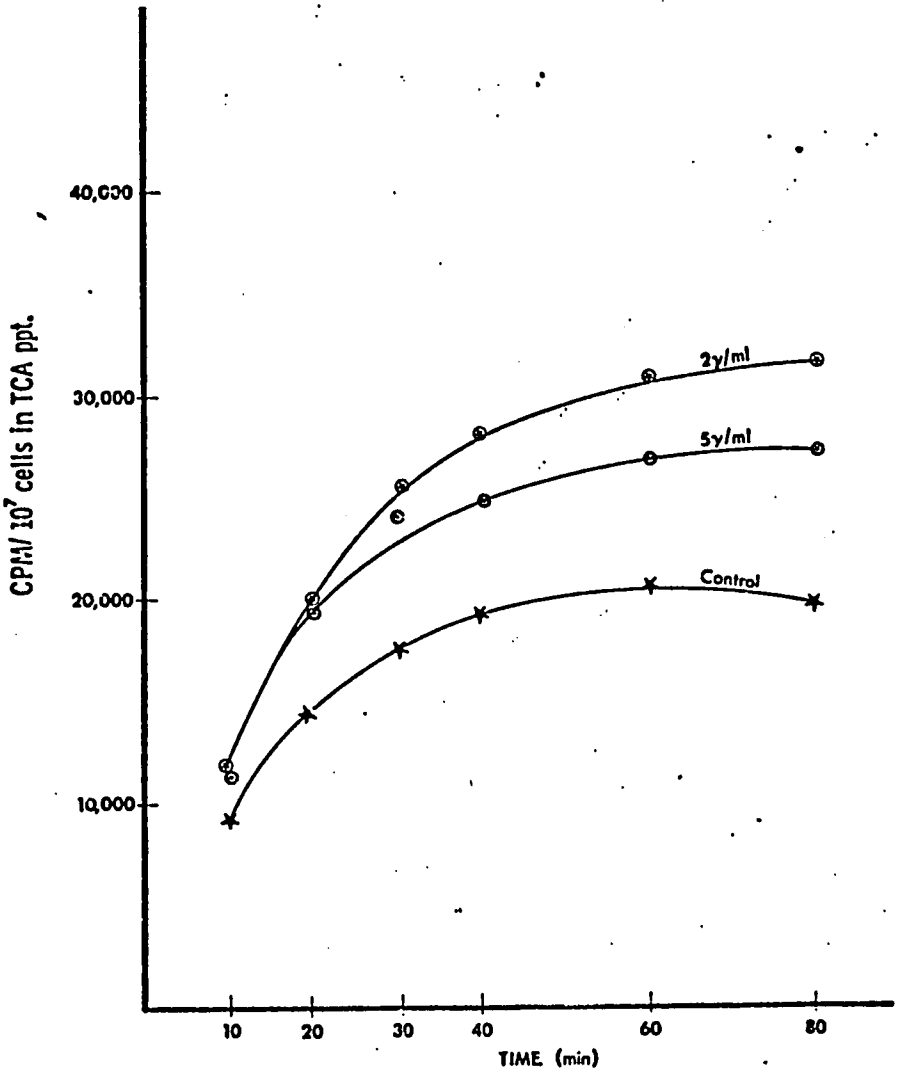

CPM in TCA ppt./ 10^7 cells
40,000
30,000
20,000
10,000
20γ/ml
10γ/ml
Control
10
20
30
40
60
90
TIME (min)

Figure 21: Effect of Berenil 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 Berenil (2µg/ml and 5µg/ml).


40,000
30,000
20,000
10,000
CPM/ 10^7 cells in TCA ppt.
2γ/ml
5γ/ml
Control
10
20
30
40
60
80
TIME (min)

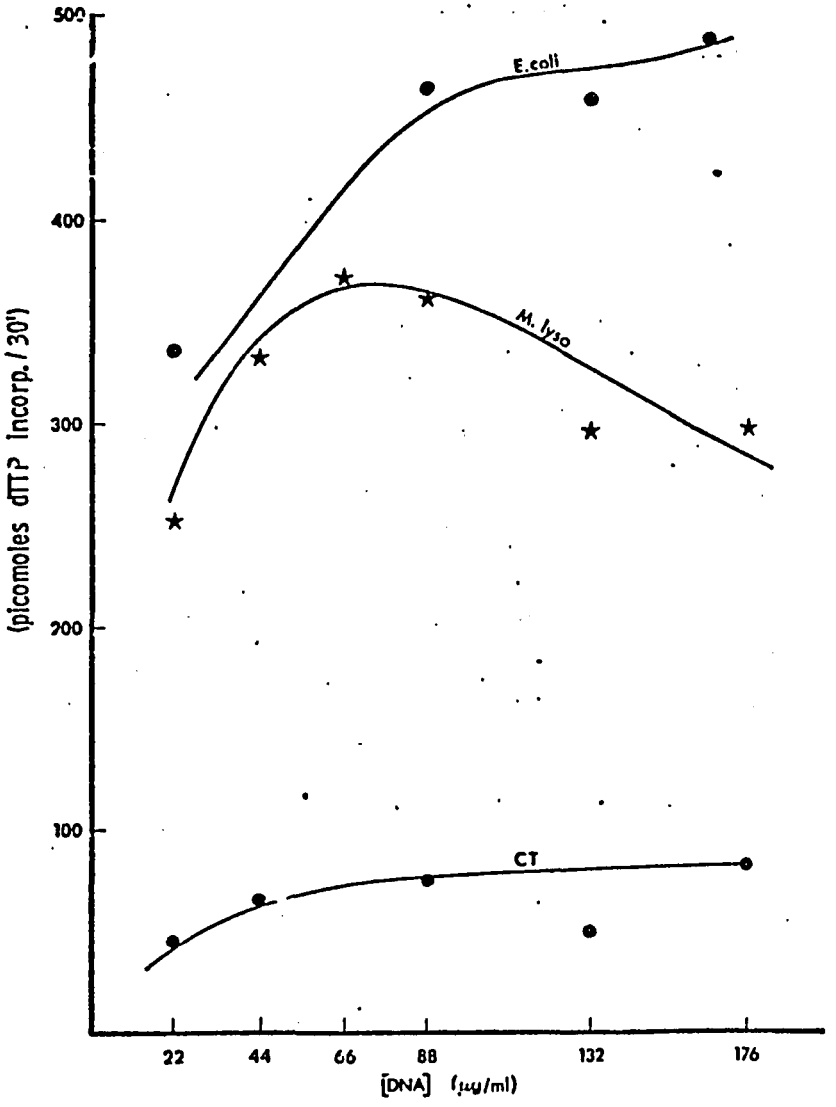
Effect of Berenil on DNA Polymerase

The effect of Berenil on the DNA polymerase-catalyzed incorporation of ^{3}H-dTTP into DNA was examined using three different DNA polymerases, two bacterial and one mammalian. Unless otherwise mentioned denatured CT-DNA was used as template in all the assays. Omission of DNA or any of the polymerases from the standard reaction mixture reduced the amount of DNA synthesized by 98%. Omission of one nucleoside 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 optimum Mg^{++} concentration is 8-10 mM, and the radioactivity incorporated was sensitive to DNAase.

The DNA dependence of the reaction for each polymerase is shown in Figure 22. In this system DNA is at a saturating level for the E. coli enzyme at ∿100 µg/ml, for the M. lysodeikticus enzyme at ∿66 µg/ml and for the calf thymus enzyme at ∿88 µg/ml.

Figure 23 indicates that for all three polymerases the reaction proceeds at a constant rate for at least 90 minutes. The reaction was stopped at the appropriate times by pipetting the reaction mixture onto filter paper disks, previously warmed to 50°C. The reaction may also be stopped by the addition of Berenil (100 µg/ml) to the reaction mixture. The time dependence experiment illustrated in Figure 23 was repeated using Berenil to stop the reaction at the appropriate times as follows: Ten standard assays for each of the three enzymes were started simultaneously. At 15, 30, 45, 60 and 90 minutes Berenil was added to two each of the ten tubes. After addition of Berenil, the 37°C incubation was continued until the last reaction was stopped at 90 minutes. At this time, the contents of all tubes were pipetted onto filter paper disks.

Figure 22: DNA dependence of the three DNA polymerase reactions. All polymerases were present at a concentration of 5 U/ml in the standard reaction mixture.


500
400
300
200
100
(picomoles dTTP Incorp./30')
E.coli
M. lyso
CT
22
44
66
88
132
176
[DNA] (µg/ml)

The results of this experiment were the same as those shown in Figure 23. This observation suggests that not only does Berenil stop DNA synthesis instantaneously but that Berenil does not cause the degradation of newly synthesized DNA.

Figure 24 reveals that Berenil inhibits the activity of all three DNA polymerases. It also suggests that the bacterial enzymes are more sensitive to inhibition by Berenil (50% inhibition is achieved at 5 μg/ml Berenil) than is the mammalian enzyme (50% inhibition is achieved at 10 μg/ml Berenil). A concentration of 5 μg/ml Berenil also inhibited the E. coli enzyme 50% when heat-denatured E. coli DNA was used in the reaction mixture instead of heat-denatured CT-DNA.

To determine the Berenil-sensitive constituent of the enzyme reaction, the experiments described in Figures 25-27 were performed. The ability of either additional enzyme, additional DNA, or sRNA to relieve the Berenil inhibition of DNA synthesis was examined. Figures 25 and 26 reveal that in the case of bacterial enzymes, only addition of enzyme effectively overcomes the Berenil inhibition. Figure 27 reveals that the Berenil inhibition of the mammalian enzyme can be overcome by addition of either DNA or enzyme.

These results suggest that:

(1) Berenil inhibits the bacterial polymerases by direct action on the enzyme.

(2) Berenil does not destroy the template activity of DNA for the bacterial enzymes, and

(3) the mechanism of Berenil inhibition of the bacterial vs. the mammalian enzymes is different.

Attempts were made to obtain a trypanosome homogenate with DNA

Figure 23: 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.

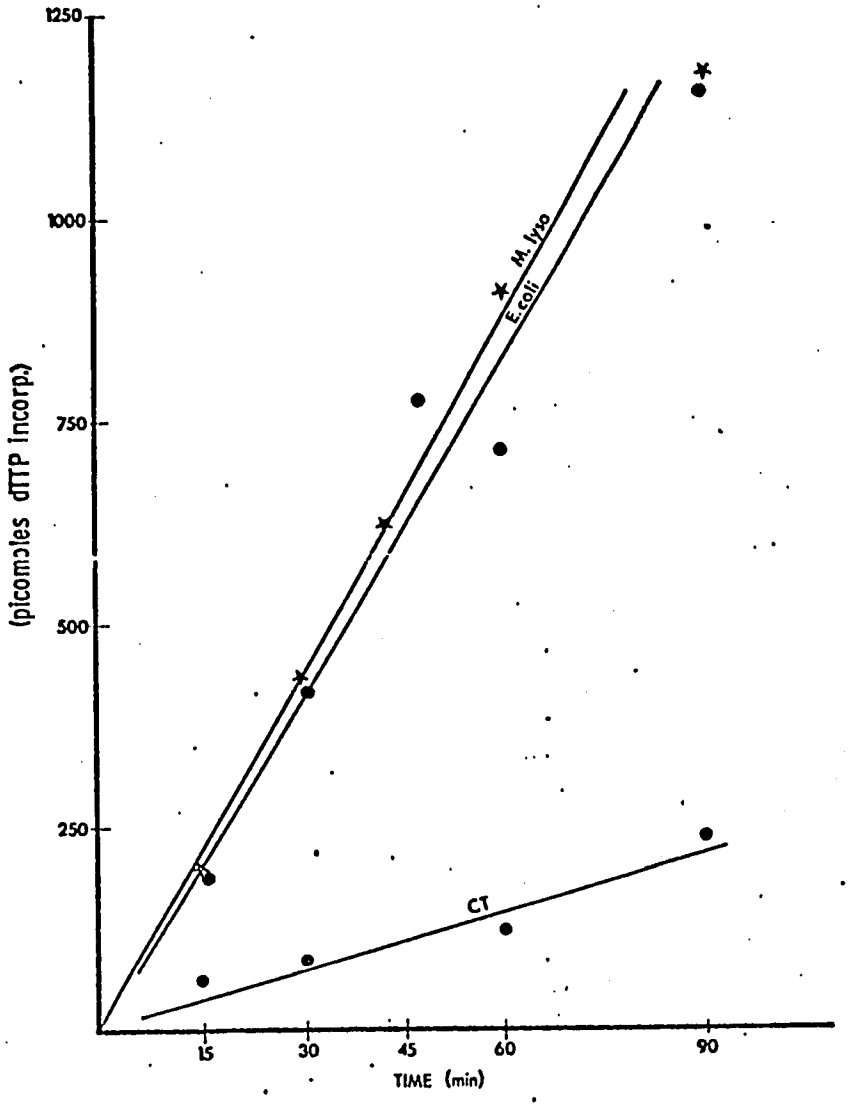

1250
1000
750
500
250
(picomoles dTTP Incorp.)
M. lyso
E.coli
CT
15
30
45
60
90
TIME (min)

Figure 24: The effect of increasing concentrations of Berenil 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.

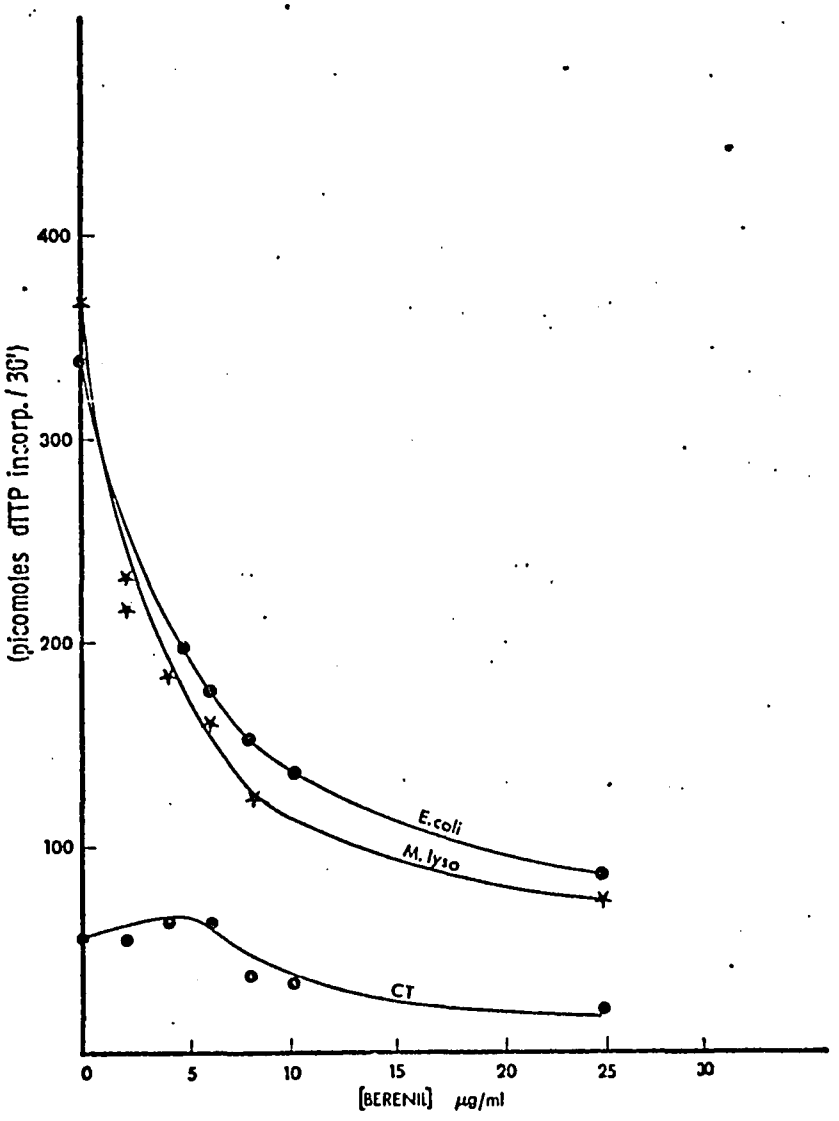

(picomoles dTTP incorp./ 30')
400
300
200
100
0
5
10
15
20
25
30
[BERENIL] µg/ml
E.coli
M. lyso
CT

Figure 25: Conditions for reversibility of Berenil inhibition of *E. coli* DNA polymerase. The effect of increasing amounts of polymerase, DNA and sRNA in a standard reaction mixture which initially contained 5μg/ml Berenil, 5 units/ml *E. coli* DNA polymerase and 176 μg/ml DNA was examined.

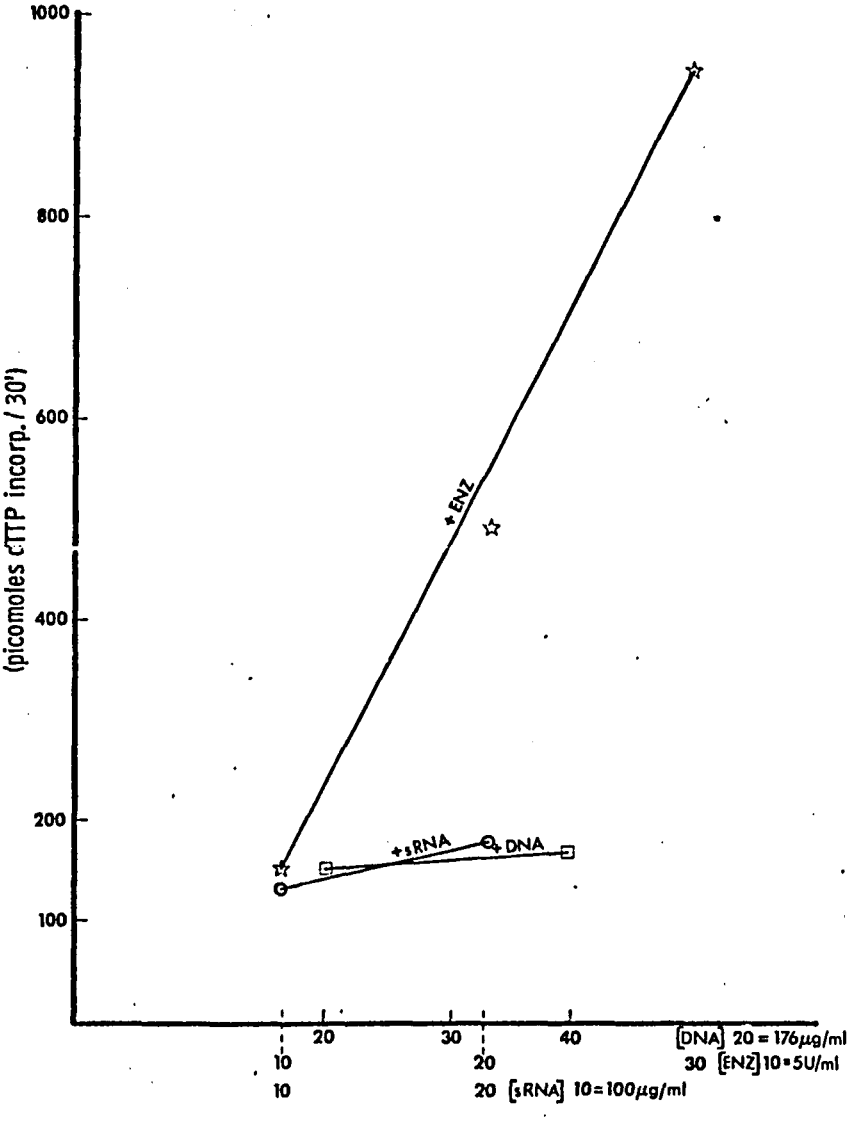

1000
800
600
400
200
100
(picomoles cTTP incorp. / 30')
+ENZ
+sRNA
DNA
20
30
40
[DNA] 20 = 176µg/ml
10
20
30 [ENZ] 10 = 5U/ml
10
20 [sRNA] 10 = 100µg/ml

Figure 26: Conditions for reversibility of Berenil inhibition of M. lysodeikticus DNA polymerase. The effect of increasing amounts of polymerase, DNA and sRNA in a standard reaction mixture which initially contained 5 μg/ml Berenil, 5 units/ml M. lysodeikticus DNA polymerase and 176 μg/ml DNA was examined.

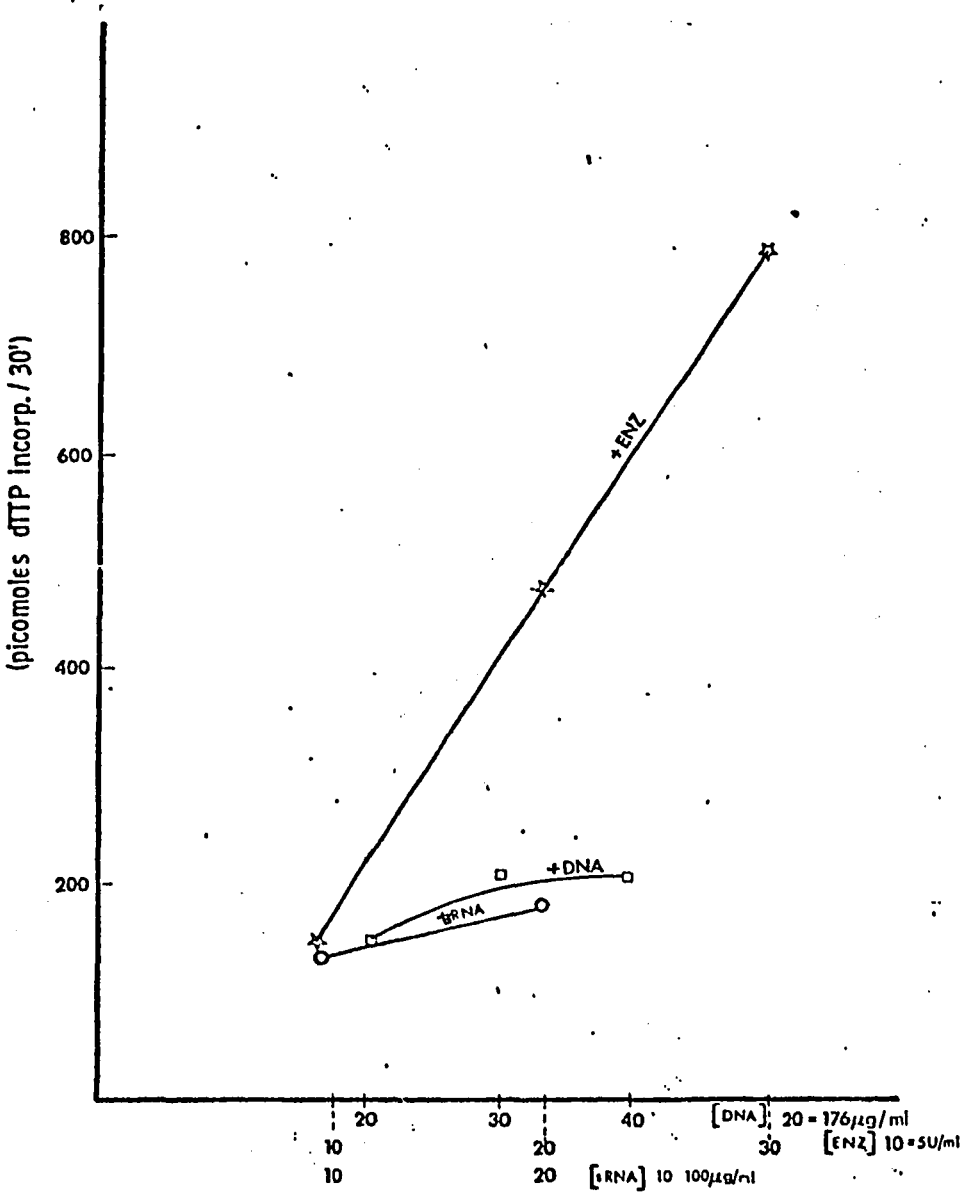
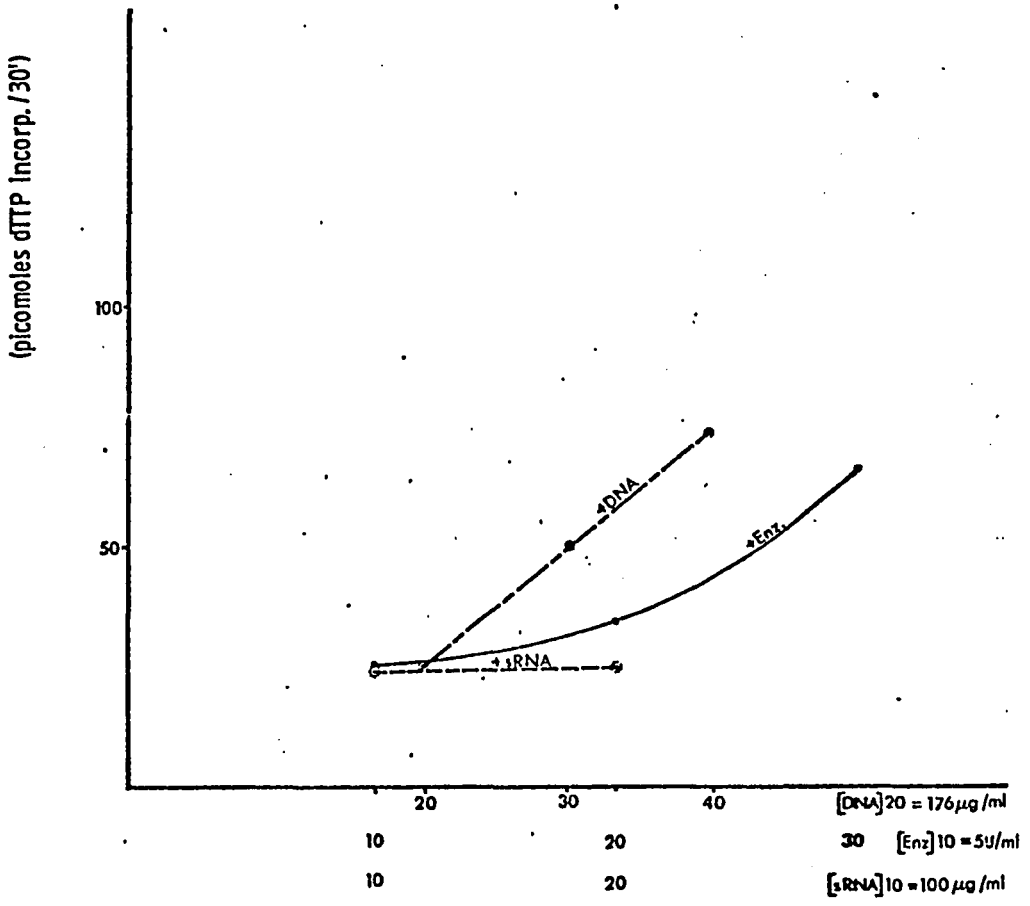

(picomoles dTTP Incorp. / 30')
800
600
400
200
+ENZ
+DNA
+tRNA
20
30
40
10
20
30
10
20
[DNA] 20 = 176µg/ml
[ENZ] 10 = 5U/ml
[tRNA] 10 100µg/ml

Figure 27: Conditions for the reversibility of Berenil inhibition of calf thymus DNA polymerase. The effect of increasing amounts of polymerase, DNA and sRNA in a standard reaction mixture which initially contained 10 μg/ml Berenil, 5 units/ml calf thymus DNA polymerase and 176 μg/ml DNA was examined.


(picomoles dTTP Incorp./30')
100
50
+DNA
+Enz.
+tRNA
20
30
40
[DNA]20 = 176µg/ml
10
20
30
[Enz]10 = 5U/ml
10
20
[tRNA]10 = 100µg/ml

synthesizing activity. The methods used to prepare the homogenate were:

(1) Purified trypanosomes were placed in a ground glass tissue grinder and ground with glass beads (at 4°C),

(2) Purified trypanosomes were swollen in a hypotonic medium (at 4°C) and forced several times through a hypodermic syringe, fitted with a #26 gauge needle (Simpson, 1968).

Both methods resulted in >70% breakage of the cells, as judged by microscopic examination. Neither procedure yielded homogenates with DNA synthesizing activity.

Effect of Berenil on RNA Polymerase

The characteristics of the RNA synthesizing system used have been described (Chamberlain and Berg, 1962). When Berenil (50μg/ml) was added to the standard reaction mixture, only a slight (<5%) inhibition of RNA synthesis was observed. Berenil at 100 μg/ml inhibited RNA synthesis by 20%. These observations indicate that the bacterial RNA polymerase (*E. coli*) used in these studies is much less sensitive to Berenil inhibition than any of the DNA polymerases studied.

DISCUSSION

Effect of Berenil on the Growth of T. brucei In Vivo

The persistence of living trypanosomes in the bloodstream of Berenil-treated mice for many hours after drug treatment suggests that Berenil does not interfere with the enzymes involved in maintenance metabolism in trypanosomes. The observation that the number of trypanosomes in the mouse bloodstream does not increase after Berenil treatment suggests that Berenil may inhibit cell division. This inference is supported by the ultrastructural alterations seen in trypanosomes exposed *in vivo* to a curative dose of Berenil for eighteen hours. At this time, a large number of what appear to be predivisional forms is apparent in the cell population. The observation that trypanosomes exposed *in vivo* to a curative dose of Berenil contain approximately twice the amount of DNA, RNA and protein usually found in these cells is further evidence that Berenil-treated trypanosomes are blocked in division. There have been no reports suggesting that Berenil acts as a mitotic inhibitor on trypanosomes or any other cells.

Effect of Berenil on Incorporation of ^{3}H-Thymidine into Trypanosome DNA

The recovery of only ^{3}H-thymine from 70% PCA hydrolysates of cold dilute acid insoluble material derived from trypanosomes labeled *in vitro* or *in vivo* with ^{3}H-Tdr, and the alkali stability of the radioactivity in 5% TCA precipitates of trypanosomes labeled with ^{3}H-Tdr suggests that ^{3}H-Tdr is incorporated into the DNA of trypanosomes.

It appears that Berenil reversibly inhibits the incorporation of ^{3}H-Tdr into trypanosome DNA under both *in vivo* and *in vitro* conditions of drug exposure. The observed inhibition of ^{3}H-Tdr incorporation into trypanosome DNA could reflect

(a) decreased permeability of the trypanosome to ^{3}H-Tdr following Berenil treatment,

(b) decreased rate of DNA synthesis following Berenil treatment, and/or

(c) enhanced rate of DNA degradation following Berenil treatment.

Although possibility (a) has not been investigated directly, the data obtained in this investigation suggest that inhibition of incorporation reflects an inhibition of synthesis. The evidence supporting this conclusion is:

(a) There is more DNA/cell after Berenil treatment, and

(b) Berenil inhibits the activity of a variety of DNA polymerases in vitro.

Effect of Berenil on Incorporation of ^{3}H-Uracil into Trypanosome RNA

The recovery of ^{3}H-cytosine, ^{3}H-uracil and a small amount of ^{3}H-thymine from 70% PCA hydrolysates of cold dilute acid insoluble material derived from trypanosomes labeled in vitro or in vivo with ^{3}H-U, and the alkali sensitivity of the radioactivity in 5% TCA precipitates of trypanosomes labeled with ^{3}H-U, suggests that ^{3}H-U is incorporated into the RNA of trypanosomes.

It appears that Berenil reversibly inhibits the incorporation of ^{3}H-U into trypanosome RNA under both in vivo and in vitro conditions of drug exposure. The inhibition of incorporation could reflect,

(a) decreased permeability of the trypanosome to ^{3}H-U following Berenil treatment,

(b) decreased rate of RNA synthesis following Berenil treatment, and/or

(c) enhanced rate of RNA degradation following Berenil treatment.

Possibility (a) has not been investigated directly. We have attempted to examine possibility (b). It appears that the activity of E. coli RNA polymerase (+ sigma factor) is not inhibited by chemotherapeutically useful concentrations of Berenil. If this enzyme can serve as a reliable model for trypanosome RNA polymerases, we may conclude that if Berenil does inhibit RNA synthesis in trypanosomes, it is probably not as a result of direct action of the drug on RNA polymerase. This point cannot be clarified until RNA polymerases are isolated from trypanosomes.

If Berenil does not inhibit RNA synthesis by direct action on RNA polymerases (if it does inhibit RNA synthesis) then we must conclude that RNA synthesis is inhibited in an indirect manner (i.e. such as unavailability of the DNA template to RNA polymerase as a result of Berenil treatment).

We have attempted to examine possibility (c) by performing some pulse and chase experiments using washed trypanosomes in vitro. Extreme caution should be used in interpreting these data since the RNA synthesized by trypanosomes in vitro and RNA synthesized in the presence of Berenil either in vitro or in vivo may be qualitatively different from the RNA synthesized under normal in vivo conditions. Figures 29-31 (see Appendix) suggest that there are quantitative differences in uracil conversions in trypanosomes in vivo and washed trypanosomes in vitro.

The pulse and chase experiments performed during the course of this study have provided the following observations:

(1) Pre-existing RNA is degraded more rapidly in the presence of Berenil than it is in the absence of Berenil

(2) RNA synthesized in the presence of Berenil is more stable than

RNA synthesized in the absence of Berenil.

Some hypotheses to account for these observations have been presented in Table III. In order to distinguish among these possibilities, it will be necessary to examine the effect of Berenil on in vitro RNA and protein synthesizing systems the constituents of which are derived from trypanosomes. Such in vitro systems have not yet been developed. In addition, the effect of Berenil on various trypanosome RNAases should be examined. Ultimately, the pulse and chase experiments should be performed on bloodstream trypanosomes growing in vitro should an appropriate cultivation medium be developed. We conclude that Berenil reversibly decreases the incorporation of ^{3}H-U into trypanosome RNA either by inhibiting RNA synthesis and/or enhancing RNA degradation.

Effect of Berenil on the Activity of DNA Polymerases In Vitro

DNA enzymology is in a somewhat confused state at the present time. It is not clear precisely which enzyme(s) is responsible for DNA replication in vivo in E. coli or any other organism (Goulian, 1972). There is good genetic evidence that E. coli DNA polymerase I functions in DNA repair in vivo, but is probably not responsible for DNA replication in vivo (Goulian, 1972).

We have examined the effect of Berenil on the activity of several DNA polymerases in vitro. These enzymes are readily available commercially. It would be interesting to examine the effect of Berenil on DNA polymerase II and III from E. coli, since these enzymes have been implicated in DNA replication (Goulian, 1972). We have determined that Berenil inhibits the activity of all DNA polyermase I's examined (the E. coli, M. lysodeikticus and calf thymus enzymes). It would be useful to know if Berenil is a general DNA polymerase inhibitor (I, II and III) or

whether it is specific for polymerase I. DNA polymerase I catalyzes several different reactions. In addition to DNA synthesis (phosphodiester bond formation, 5'→3' direction) it catalyzes two distinct exonuclease reactions, one degrades DNA in the 3'→5' direction (3'→5' exonuclease) and the other degrades DNA in the 5'→3' direction (5'→3' exonuclease) (Setlow, Brutlag and Kornberg, 1972). Our studies have demonstrated that for all three polymerases, addition of Berenil (100 μg/ml) instantaneously stops the DNA polymerase-catalyzed incorporation of ^{3}H-dTTP into DNA. In addition, we have observed that radioactivity incorporated into DNA prior to addition of Berenil is stable during prolonged (up to 90 minutes) incubation of the reaction mixture at 37°C in the presence of Berenil.

These observations suggest that Berenil does not itself cause degradation of DNA, and also suggests that Berenil does not activate the 3'→5' exonuclease activity of DNA polymerase. The effect of Berenil on the 3'→5' and 5'→3' exonuclease activities of DNA polymerase according to the method of Setlow and Kornberg (1972) have not been examined, but should be at some future time.

Our studies of the effect of Berenil on the activity of several DNA polymerase I's have revealed the following:

(a) The bacterial enzymes are more sensitive to Berenil inhibition than is the mammalian enzyme, and

(b) Berenil inhibition of the bacterial enzyme may be overcome only by the addition of DNA polymerase (i.e. binding of Berenil to DNA does not destroy the template activity of DNA for these enzymes). Berenil inhibition of the mammalian enzyme may be overcome either by addition of DNA or DNA polymerase (i.e. Berenil binding to DNA does in part destroy the template activity of DNA for calf thymus DNA polymerase). These results suggest

that:

(a) Berenil inhibits DNA polymerases by direct action on the enzyme, and

(b) The bacterial and mammalian enzymes differ in the stringency of their template requirements, the mammalian enzyme being more sensitive to distortion of the template than are the bacterial enzymes.

In order to relate this information to the effect of Berenil on DNA synthesis in trypanosomes, the effect of Berenil on DNA polymerases from trypanosomes should be investigated. The inferences obtained from the present studies may be extended to construct a hypothesis to explain the observed "kinetoplast selectivity" of Berenil. It has been reported that ethidium bromide and acriflavin selectively inhibit mitochondrial DNA polymerase over nuclear DNA polymerase in an in vitro system derived from rat liver cells. The drug sensitive constituents of the reaction systems were not examined (Meyer and Simpson, 1969). If the effect of Berenil on replicative DNA polymerases from trypanosome nuclei and mitochondria is akin to its effect on the DNA polymerase I's examined then the observed "kinetoplast selectivity" of Berenil might be explained on the basis of greater Berenil sensitivity of the mitochondrial enzyme. It may be that the progressive disorganization of the KP-DNA seen after Berenil treatment results from stimulation of exonuclease activity, either that apart from or that associated with a mitochondrial DNA polymerase I.

Berenil Cure of Experimental Trypanosomiasis

The experiments performed during the course of this study have yielded the following observations:

(a) a single injection of Berenil (10-100 µg/1g body weight) cures mice of T. brucei (monomorphic) infection.

(b) Trypanosomes isolated from Berenil-treated mice appear to be blocked in division.

(c) Berenil causes a temporary and reversible inhibition of DNA (and possibly RNA) synthesis in trypanosomes.

It is not possible, from these data, to determine whether the inhibition of DNA (and possibly RNA) synthesis is the cause of the blockage in cell division and in turn whether the blockage of cell division is the primary cause of the lethal action of Berenil on T. brucei. Hence, the following hypotheses may be advanced:

(1) The lethal action of Berenil on T. brucei is unrelated to its ability to block cell division, and results from some activity of the drug not yet understood.

(2) The lethal action of Berenil on T. brucei is related to its ability to block cell divison, but it does so by some means not yet understood. Berenil inhibition of DNA synthesis is not related to its ability to block cell division.

(3) The lethal action of Berenil on T. brucei is related to its ability to temporarily inhibit DNA (and possibly RNA) synthesis. Temporary inhibition of DNA (and possibly RNA) synthesis (perhaps through induction of a state of unbalanced growth) permanently destroys the synchrony of the cell cycle and renders trypanosomes unable to divide. This results in:

(a) elimination of trypanosomes by the host (perhaps through an immunological process) or

(b) continued growth of the trypanosomes until the mass to surface ratio exceeds a certain critical value, and the trypanosomes lyse.

Hypotheses (1) and (2) are possibilities which should be considered. It is possible that Berenil acts by an as yet unelucidated mechanism. Hypothesis (3a) has been suggested to explain the mechanism of action of many of the anticancer agents which are inhibitors of DNA synthesis (Helmstetter, 1971). It has been suggested that neoplastic cells find themselves in a hostile environment and that the real effect of many anticancer agents may be to slow down the rate of replication of neoplastic cells so that other processes, perhaps immunological in nature, can more effectively remove the cells. Thus, cells although not actually killed by unbalanced browth may be rendered more sensitive by it to other environmental factors. This line of reasoning may be applied to the effect of Berenil on trypanosomes. Some evidence has been presented suggesting that the host reticulo-endothelial system participates in the chemotherapeutic activity of the trypanocides Antrycide and Suramin (Sen, Dutta and Ray, 1955). The same may be true for Berenil.

Hypothesis (3b) differs from (3a) in that it suggests that unbalanced growth actually kills the trypanosomes. It might be possible to distinguish between these two hypotheses by observing the course of Berenil cure in infected hosts that have been treated with immunosuppressants. It might also be useful to examine the effect of splenectomy on the course of Berenil cure. Such experiments could determine the extent of participation of the host immune system in Berenil cure, and thus distinguish between hypotheses (3a) and (3b).

This study was undertaken to elucidate the mechanism of action of Berenil in curing African trypanosomiasis. As a result of observations made during the course of this study, some testable hypotheses have been advanced to explain the mechanism of action of Berenil, and future work

will be directed toward distinguishing among these hypotheses. In addition, this study has added to our understanding of the biochemical capabilities of bloodstream trypanosomes by exploring the ability of these organisms to synthesize DNA, RNA and protein outside the host bloodstream under minimal *in vitro* conditions of maintenance. The results obtained in this study underscore the importance of using dual evaluation systems (*in vitro* and *in vivo* systems) for investigating the mode of action of trypanocidal drugs.

SUMMARY

We have employed a rodent-adapted, laboratory strain of Trypanosoma brucei (monomorphic) to examine the effect of Berenil on growth and nucleic acid synthesis in bloodstream trypanosomes. Our data suggest that Berenil interferes with the growth of trypanosomes in the mouse bloodstream. We conclude that Berenil blocks cell division in trypanosomes. The evidence for this conclusion is:

(1) No increase in trypanosome numbers is observed in the mouse bloodstream after a single injection of a curative dose of Berenil is administered to an infected mouse. Only a slow decrease in cell number is observed.

(2) Berenil treatment of infected mice results in an accumulation of what appear to be predivisional forms of trypanosomes in the mouse bloodstream.

(3) Trypanosomes isolated from infected Berenil-treated mice twelve hours after drug treatment contain approximately twice the amount of DNA, RNA and protein usually found in these cells.

We conclude that Berenil inhibits DNA synthesis in trypanosomes. The evidence for this conclusion is:

(1) Berenil reversibly inhibits the incorporation of ^{3}H-thymidine into the DNA of trypanosomes whether these cells are growing in the host bloodstream, or are suspended in a buffered salts-glucose solution.

(2) Berenil reversibly inhibits the activity of a variety of DNA polymerases.

We conclude that Berenil interferes with RNA metabolism in trypanosomes either by inhibiting RNA synthesis and/or enhancing the rate of RNA degradation. The evidence for this conclusion is:

(1) Berenil reversibly inhibits the incorporation of ^{3}H-uracil into the RNA of trypanosomes whether these cells are growing in the host bloodstream, or are suspended in a buffered salts-glucose solution.

(2) Berenil treatment appears to enhance the degradation of pre-existing RNA in trypanosomes.

(3) RNA synthesized by trypanosomes during exposure to Berenil appears to be more stable than RNA synthesized in the absence of Berenil.

(4) Berenil does not inhibit E. coli RNA polymerase at chemotherapeutically useful concentrations.

It is not possible at this time either on the basis of these data or on the basis of any other available data to determine whether inhibition of DNA (and possibly RNA) synthesis is the cause of the block in cell division in trypanosomes and in turn whether the blockage of cell division is the primary cause of the lethal action of Berenil on T. brucei. If the lethal action of Berenil on T. brucei is related to its ability to temporarily inhibit DNA (and possibly RNA) synthesis then perhaps this inhibition serves to destroy the synchrony of the cell cycle, rendering the trypanosomes unable to divide. Division-blocked trypanosomes may either continue to grow until they lyse, or are perhaps eliminated through an immunological process. This hypothesis is readily testable and will be challenged in future studies.

APPENDIX

Chromatography of Perchloric Acid Hydrolysates from Trypanosomes

One Dimensional Chromatograms

Figure 28 depicts the radioactive bases derived from nucleic acids of trypanosomes 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. 29) 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. 30 and 31) 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 IV. The R_f values of the five nucleic acid bases in the two solvent systems are shown in Table V. The data obtained from the chromatograms suggests that

(1) The metabolic fate of thymidine *in vivo* and *in vitro* is the same.

(2) When trypanosomes are incubated with uracil under *in vitro* conditions much less of the uracil is converted to cytosine and more uracil is converted to thymine, than when trypanosomes are *in vivo*.

Figure 28: 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.

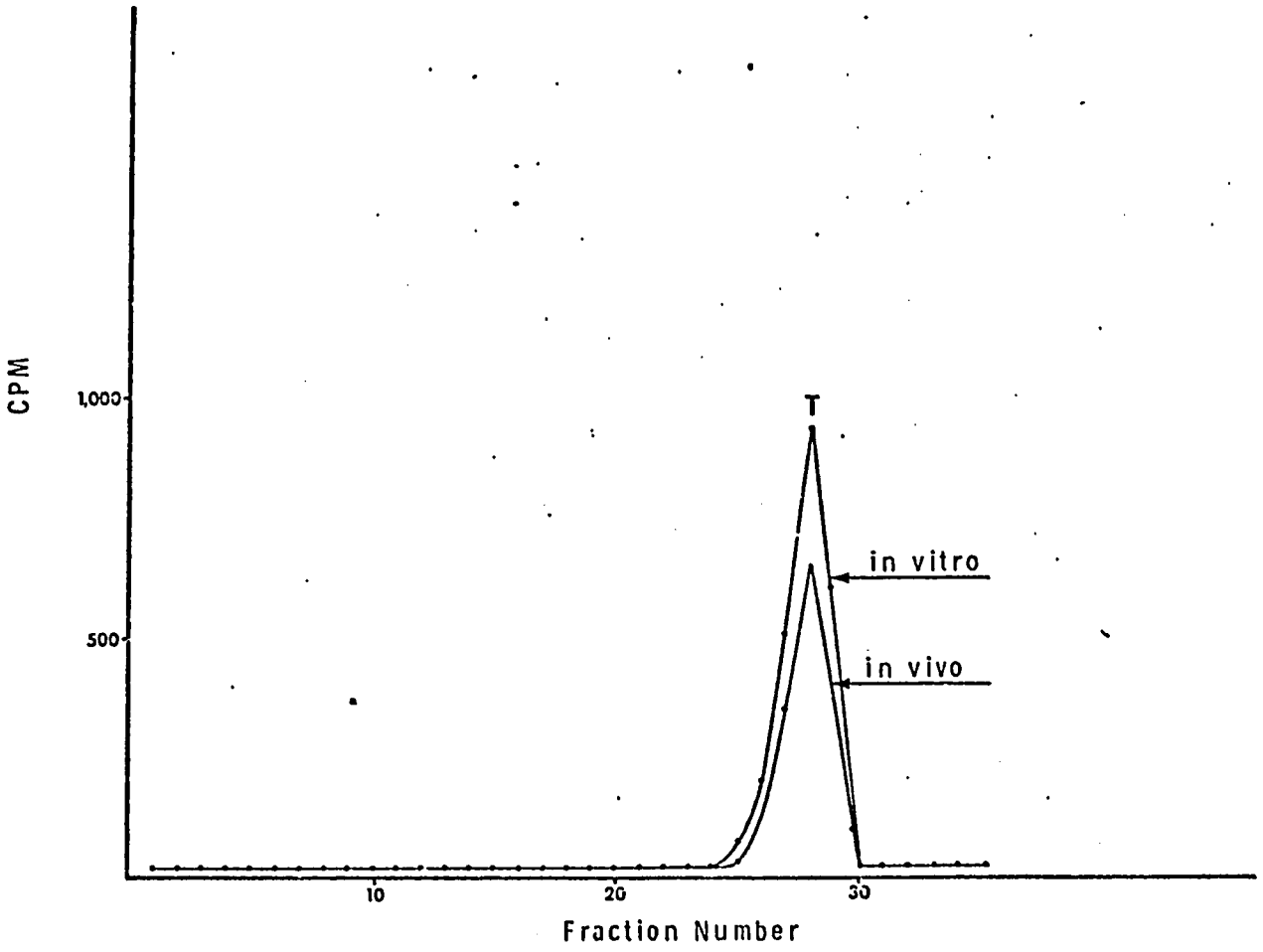

CPM
1,000
500
T
in vitro
in vivo
10
20
30
Fraction Number

Figure 29: Radioactivity profile obtained from paper chromatogram of PCA hydrolysate of trypanosomes labeled in vivo with ^{3}H-uracil (2 hours, 0.5mCi/mouse). The chromatogram was developed for 24 hours at room temperature in solvent 1.

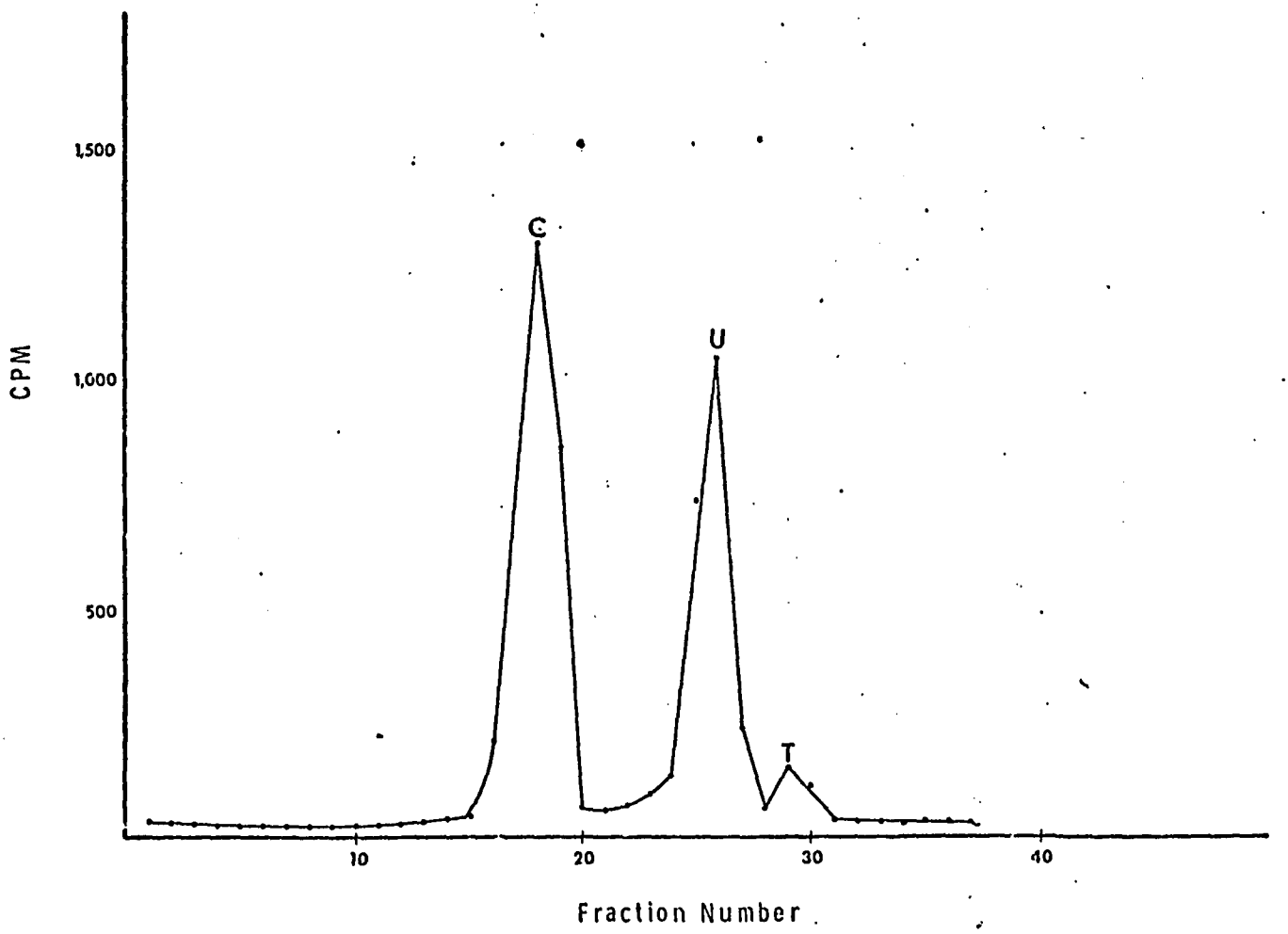

1,500
1,000
500
CPM
C
U
T
10
20
30
40
Fraction Number

Figure 30: 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.

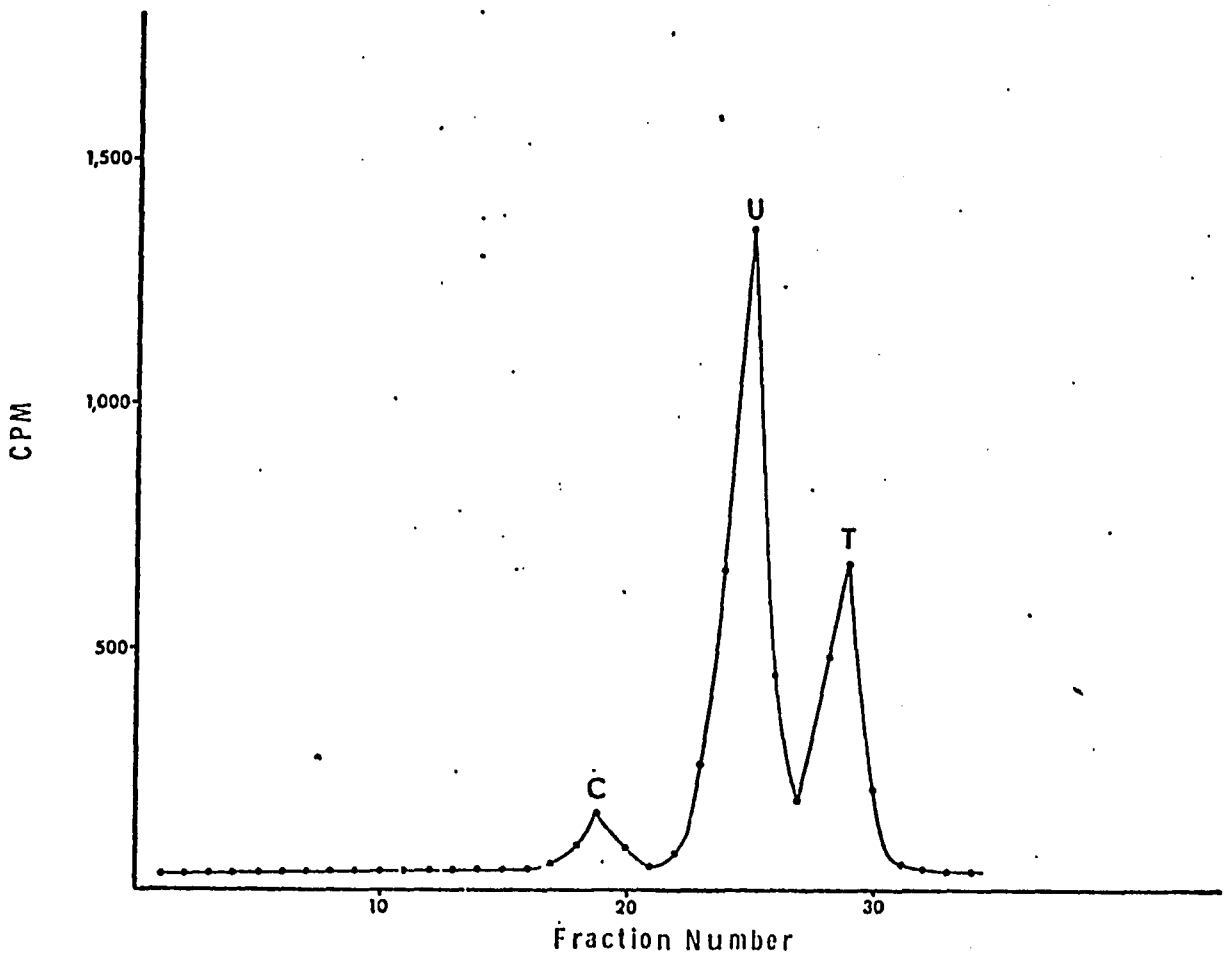

1,500
1,000
500
CPM
U
T
C
10
20
30
Fraction Number

Figure 31: Radioactivity profile obtained from paper chromatogram of PCA hydrolysate of trypanosomes labeled in vitro with ^{14}C-uracil. Same conditions as Fig. 30, except labeling was performed at 37°C.

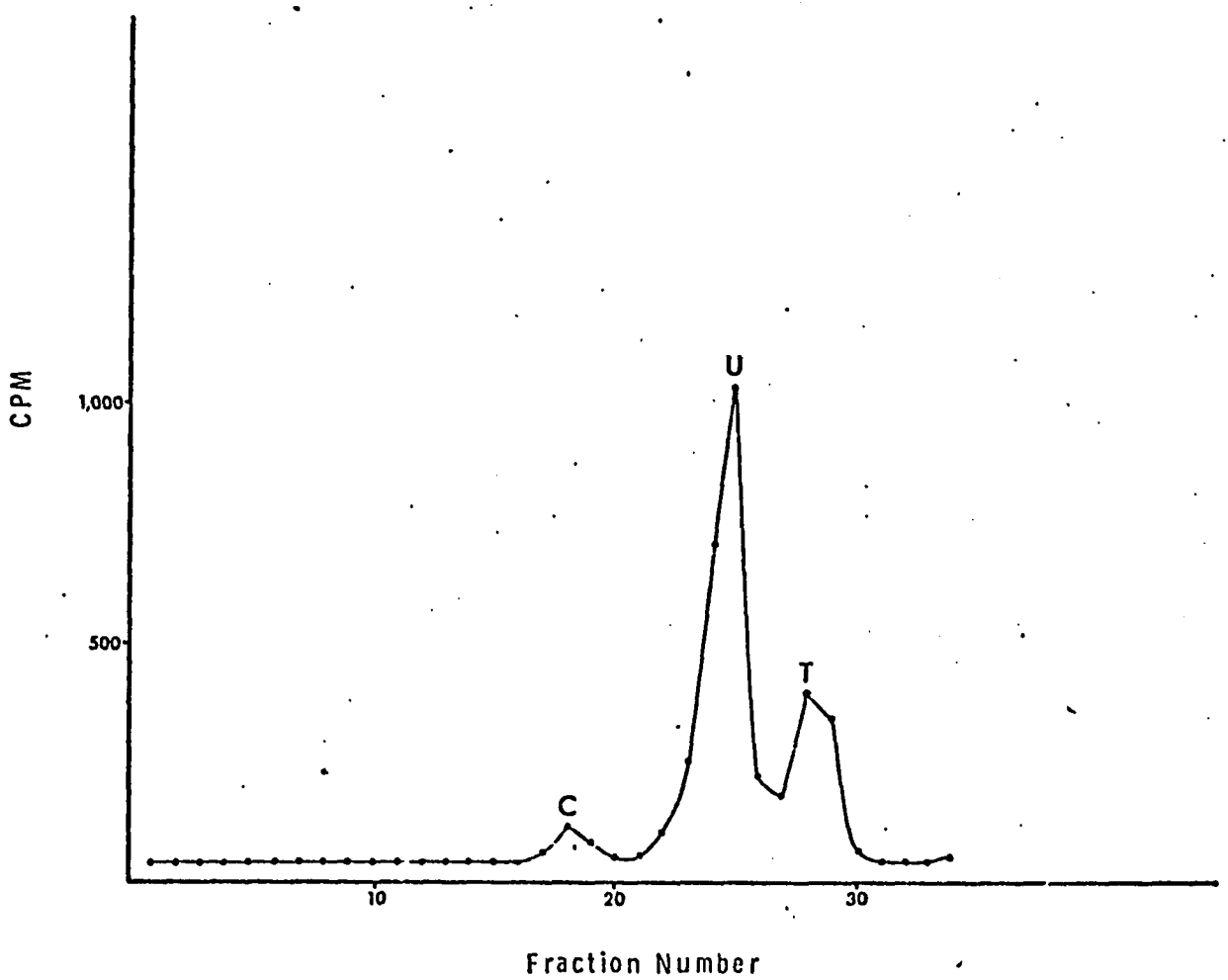

CPM
1,000
500
C
U
T
10
20
30
Fraction Number

TABLE IV

Location of Radioactivity in Two Dimensional Chromatograms

Base	Radioactivity from cells labeled with ^{3}H-U (cpm)	Radioactivity from cells labeled with ^{3}H-Tdr (cpm)
G	47	43
A	34	38
C	368	46
U	380	35
T	61	315

TABLE V

R_f values of Nucleic Acid Bases

Base	R_f value in Solvent 1	R_f value in Solvent 2
G	0.17	0.19
A	0.30	0.43
C	0.46	0.45
U	0.70	0.53
T	0.79	0.63

The fact that the appropriate radioactive bases are recoverable from hot 70% PCA hydrolysates of the cold dilute acid precipitable material from purified trypanosomes indicates that the nucleic acid precursors, thymidine and uracil, were incorporated into the DNA and RNA of trypanosomes under both *in vivo* conditions of growth and *in vitro* conditions of maintenance.

BIBLIOGRAPHY

Allison, A. C. (1968). The role of lysosomes in the action of drugs and hormones. Adv. Chemotherap. 3, 253.

Allsopp, B. A., A. R. Njogu and K. C. Humphryes (1971). Nature and location of Trypanosoma brucei subgroup exoantigen and its relationship to 4S antigen. Exp. Parasit. 29, 271.

Ashcroft, M. T. (1957). The polymorphism of Trypanosoma brucei and T. rhodesiense, its relation to relapses and remissions of infections in white rats, and the effect of cortisone. Ann. Trop. Med. Parasitol. 51, 301.

Ashcroft, M. T. (1960). A comparison between a syringe-passaged and a tsetse fly-transmitted line of a strain of Trypanosoma rhodesiense. Ann. Trop. Med. Parasitol. 53, 147.

Attardi, B. and G. Attardi (1971). Expression of the mitochondrial genome in HeLa cells. J. Mol. Biol. 55, 231.

Balber, A. (1972). Trypanosoma brucei: Fluxes of the morphological variants in intact and X-irradiated mice. Exp. Parasit. 31, 307.

Bollom, F. J. (1966). Filter paper disk techniques for assaying radioactive macromolecules, in Cantoni, G. L. and D. R. Davies (eds.), Procedures in Nucleic Acid Research. Harper and Row, New York. 269.

Bowman, I. B. R. and I. W. Flynn (1968). Metabolic properties and arsenical sensitivity of polymorphic brucei subgroup trypanosomes. VIIIth Inter. Congr. Trop. Med. and Malaria, Teheran. Abstr., 315.

Brack, Ch., E. Delain and G. Riou (1972a). Replicating, covalently closed, circular DNA from kinetoplasts of Trypanosoma cruzi. Proc. Nat. Acad. Sci. (USA) 69, 1642.

Brack, Ch., E. Delain, G. Riou, and B. Festy (1972b). Molecular organization of the kinetoplast DNA of Trypanosoma cruzi treated with Berenil, a DNA interacting drug. J. Ultrastruct. Res. 39, 568.

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

Chamberlain, M. and P. Berg (1962). Deoxyribonucleic acid-directed synthesis of ribonucleic acid by an enzyme from Escherichia coli. Proc. Nat. Acad. Sci. (USA) 48, 81.

Delain, E., Ch. Brack, G. Riou and B. Festy (1971). Ultrastructural alterations of Trypanosoma cruzi kinetoplast induced by the interaction of a trypanocidal drug (Hydroxystilbamidine) with the kinetoplast DNA. J. Ultrastruct. Res. 37, 200.

Desowitz, R. S. (1960). Denaturant effect of basic trypanocidal drugs on the protein of cell-free trypanosomal extracts. Exp. Parasit. 9, 233.

Elliott, W. H. (1963). The effects of antimicrobial agents on deoxyribonucleic acid polymerase. Biochem. J. 86, 562.

Festy, B., A. M. Lallemant, G. Riou, C. Brack and E. Delain (1970a). Méchanisme d'action des diamidines trypanocides. Importance de la composition en bases dans l'association Berenil-polynucleotide. Comptes Rendu 271, 684.

Festy, B., A. M. Lallemant, G. Riou and E. Delain (1970b). Mode d'action des diamidines trypanocides. Association du Berenil aux acides nucléiques in vitro. Comptes Rendu 271, 730.

Flynn, I. W. and I. B. R. Bowman (1970). Comparative biochemistry of monomorphic and pleomorphic strains of Trypanosoma rhodesiense. Trans. Roy. Soc. Trop. Med. Hyg. 64, 175.

Fulton, J. D. and D. F. Spooner (1959). Terminal respiration in certain mammalian trypanosomes. Exp. Parasit. 8, 137.

Gottlieb, M. (1972). Ph.D. Thesis, City University of New York, New York.

Goulian, M. (1972). Some recent developments in DNA enzymology, in Davidson, J. N. and W. E. Cohn (eds.), Progress in Nucleic Acid Research and Molecular Biology, Vol. XII. Academic Press, New York. 29.

Grant, P. T. (1966). Selective inhibition of energy yielding reactions. Symp. Soc. Gen. Microbiol. 16, 281.

Grant, P. T. and J. R. Sargent (1960). Properties of L-α-glycerophosphate oxidase and its role in the terminal respiration of Trypanosoma rhodesiense. Biochem. J. 76, 229.

Grant, P. T. and J. R. Sargent (1961). L-α-glycerophosphate dehydrogenase, a component of an oxidase system in Trypanosoma rhodesiense. Biochem. J. 81, 206.

Grant, P. T., J. R. Sargent, and J. F. Ryley (1961). Respiratory systems in the Trypanosomatidae. Biochem. J. 81, 200.

Gray, A. R. (1962). The influence of antibody on serological variation in Trypanosoma brucei. Ann. Trop. Med. Parasitol. 56, 4.

Gutteridge, W. E., K. Stuart, D. H. Williamson and K. Vickerman (1971). Analysis of DNA from some dyskinetoplastic species of trypanosome. Trans. Roy. Soc. Trop. Med. Hyg. 65, 247.

Hawking, F. (1963). Chemotherapy of Trypanosomiasis, in Schnitzer, R. J.

and F. Hawking (eds.) Experimental Chemotherapy, Vol. I. Academic Press, New York. 131.

Helmstetter, C. E. (1971). Coordination between chromosome replication and cell division: Cellular response to inhibition of DNA synthesis, in Mihich, E. (ed.), Drugs and Cell Regulation. Academic Press, New York. 1.

Hill, G. C. and W. A. Anderson (1970). Electron transport systems and mitochondrial DNA in trypanosomatidae: A review. Exp. Parasit. 28, 356.

Hoare, C. A. (1964). Morphological and taxonomic studies on mammalian trypanosomes. X. Revision of the systematics. J. Protozool. 11, 200.

Hoare, C. A. (1970). Systematic description of the mammalian trypanosomes of Africa, in Mulligan, H. W. (ed.) The African Trypanosomiases. George Allen and Unwin, Ltd., London. 125.

Kahan, D., A. C. Zahalsky and S. H. Hutner (1968). Protein synthesis in cell-free preparations of Crithidia fasciculata. J. Protozool. 15, 385.

Killick-Kendrick, R. (1964). The apparent loss of the kinetoplast of Trypanosoma evansi after treatment of an experimentally infected host with Berenil. Ann. Trop. Med. Parasitol. 58, 481.

Lanham, S. (1968). Separation of trypanosomes from the blood of infected rats and mice by anion-exchangers. Nature 218, 1273.

Littlefield, J. W. and D. B. Dunn (1958). The occurence and distribution of thymine and three methylated adenine bases in ribonucleic acids from several sources. Biochem. J. 70, 642.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall (1951).

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

Luft, J. H. (1961). Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. 9, 409.

MacAdam, R. F. and J. Williamson (1969). Lesions in the fine structure of Trypanosoma rhodesiense specifically associated with drug treatment. Trans. Roy. Soc. Trop. Med. Hyg. 63, 421.

MacAdam, R. F. and J. Williamson (1972). Drug effects on the fine structure of Trypanosoma rhodesiense: Diamidines. Trans. Roy. Soc. Trop. Med. Hyg. 66, 897.

Mahler, H. R. and K. Dawidowicz (1973). Autonomy of mitochondria in Saccharomyces cerevisiae in their production of messenger RNA. Proc. Nat. Acad. Sci. (USA) 70, 111.

Mendez, Y. and B. M. Honigberg (1972). Infectivity of Trypanosoma brucei subgroup flagellates maintained in culture. J. Parasit. 58, 1122.

Meyer, R. R. and M. V. Simpson (1969). Differential inhibition of mitochondrial and nuclear DNA polymerases by the mutagenic dyes Ethidium bromide and acriflavin. Biochem. Biophys. Res. Commun. 34, 238.

Munro, H. N. and A. Fleck (1966). The determination of nucleic acids, in Glick, D. (ed.) Methods of Biochemical Analysis, Vol. XIV. J. Wiley and Sons, New York. 113.

Newton, B. A. (1957). Nutritional requirements and biosynthetic capabilities of the parasitic flagellate Strigomonas oncopelti. J. Gen. Microbiol. 17, 708.

Newton, B. A. (1966). Effects of antrycide on nucleic acid synthesis

and function. Symp. Soc. Gen. Microbiol 16, 213.

Newton, B. A. (1967). Interaction of Berenil with deoxyribonucleic acid and some characteristics of the Berenil-DNA complex. Biochem. J. 105, 50p.

Newton, B. A. (1972). Recent studies on the mode of action of Berenil, in Van Den Boosche, H. (ed.), Comparative Biochemistry of Parasites (Proceedings of Int. Symp. Beerse, Belg. 1971). Academic Press, New York. 127.

Newton, B. A. and R. W. F. LePage (1967). Preferential inhibition of extranuclear deoxyribonucleic acid synthesis by the trypanocide Berenil. Biochem. J. 105, 50p.

Newton, B. A.and R. W. F. LePage (1968). Interaction of Berenil with trypanosome DNA. Trans. Roy. Soc. Trop. Med. Hyg. 62, 131.

Newton, B. A. and J. K. Burnett (1971). The heterogeneity of trypanosome DNA. Trans. Roy. Soc. Trop. Med. Hyg. 65, 243.

Oehler, R. (1914). Untersuchungen über den dimorphismus von Trypanosoma brucei. Z. Hyg. Infektionskrankh. 77, 356.

Ono, T. Y. Ozeki, S. Okubo, and S. Inoki (1971). Characterization of nuclear and satellite DNA from trypanosomes. Biken J. 14, 203.

Ozeki, Y., V. Sooksri, T. Ono and S. Inoki (1971). Studies on the ultrastructure of kinetoplast of Trypanosoma cruzi and Trypanosoma gambiense by autoradiography and enzymatic digestion. Biken J. 14, 97.

Ray, H. N. and M. N. Malhotra (1960). Akinetoplastic strain of Trypanosoma evansi produced by Prothidium. Nature 188, 870.

Reichenow, E. (1921). Untersuchungen ueber das Verhalten von

Trypanosoma gambiense in menschlichen Korper. Z. Hyg. Infektionskrankh . 94, 266.

Reichenow, E. (1932). Das Verhalten von Trypanosoma gambiense in der Kultur. Z. Parasitenk. 4, 784.

Riou, G. (1967). Induction d'une dyskinetoplastie par le bromure d'éthidium chez Trypanosoma cruzi cultivé in vitro. Comptes Rendu 265, 2004.

Riou, G. and E. Delain (1969). Electron microscopy of the circular kinetoplastic DNA from Trypanosoma cruzi. Occurence of catenated forms. Proc. Nat. Acad. Sci (Washington) 62, 210.

Riou, G. and R. Pautrizel (1969). Nuclear and kinetoplastic DNA from trypanosomes. J. Protozool. 16, 509.

Rudzinska, M. A. and K. Vickerman (1968). The fine structure, in Weinman, D. and M. Ristic (eds.), Infectious Blood Diseases of Man and Animals, Vol. I. Academic Press, New York. 217.

Ryley, J. F. (1956). Studies on the metabolism of Protozoa. 7. Comparative carbohydrate metabolism of eleven species of trypanosomes. Biochem. J. 62, 215.

Ryley, J. F. (1962). Studies on the metabolism of the Protozoa. 9. Comparative metabolism of bloodstream and culture forms of Trypanosoma rhodesiense. Biochem. J. 85, 211.

Ryley, J. F. (1966). Histochemical studies on blood and culture forms of Trypanosoma rhodesiense, in Proc. 1st Intern. Congr. Parasit. 1, 41.

Schneider, W. C. (1957). Determination of Nucleic Acids in Tissues by Pentose Analysis, in Colowick, S. P. and N. O. Kaplan (eds.) Methods in Enzymology, Vol. III. Academic Press, New York. 680.

Setlow, P. and A. Kornberg (1972). Deoxyribonucleic acid polymerase: Two distinct enzymes in one polypeptide. J. Biol. Chem. 247, 232.

Setlow, P., D. Brutlag and A. Kornberg (1972). Deoxyribonucleic acid polymerase: Two distinct enzymes in one polypeptide. J. Biol. Chem. 247, 224.

Simpson, L. (1968). Behavior of the kinetoplast of Leishmania tarentolae upon cell rupture. J. Protozool. 15, 132.

Simpson, L. and A. DaSilva (1971). Isolation and characterization of kinetoplast DNA from Leishmania tarentolae. J. Mol. Biol. 56, 443.

Steiger, R. (1971). Some aspects of the surface coat formation in Trypanosoma brucei. Acta Trop. 28, 341.

Steinert, M. (1960). Mitochondria associated with the kinetonucleus of Trypanosoma mega. J. Biophys. Biochem. Cytol. 8, 542.

Steinert, M. (1965). L'Absence d'histone dans le kinetonucleus des trypanosomes. Exp. Cell Res. 39, 69.

Steinert, M., S. Van Assel and G. Steinert (1969). Étude par autoradiographie, des effets du bromure d'éthidium sur la synthèse des acides nucléique de Crithidia luciliae. Exp. Cell Res. 56, 69.

Smeesters, C. and P. J. Jacques (1968). Influence of injected suramin on enzymic equipment of rat liver lysosomes in vivo. (XII Int. Congress Cell Bio. Brussels, 1968.) Excerpta Medica Série intern. No. 166 Abst. 143, 82.

Stuart, K. D. (1971). Evidence for the retention of kinetoplast DNA in an acriflavine-induced dyskinetoplastic strain of Trypanosoma

brucei which replicates the altered central element of the kinetoplast. J. Cell. Biol. 49, 189.

Trager, W. and M. A. Rudzinska (1964). The riboflavin requirement and the effects of acriflavin on the fine structure of the kinetoplast in Leishmania tarentolae. J. Protozool. 11, 133.

Vickerman, K. (1962). The mechanism of cyclical development in trypanosomes of the Trypanosoma brucei subgroup: An hypothesis based on ultrastructural observations. Trans. Roy. Soc. Trop. Med. Hyg. 56, 487.

Vickerman, K. (1965). Polymorphism and mitochondrial activity in sleeping sickness trypanosomes. Nature 208, 762.

Vickerman, K. (1971). Morphological and physiological considerations of extracellular protozoa, in Fallis, A. M. (ed.), Ecology and Physiology of Parasites. University of Toronto Press, Toronto. 59.

Vickerman, K. and A. G. Luckins (1969). Localization of variable antigens in the surface coat of Trypanosoma brucei using ferritin conjugated antibody. Nature 224, 1125.

Vickerman, K. and T. M. Preston (1970). Spindle microtubules in the dividing nuclei of trypanosomes. J. Cell. Sci. 6, 365.

Walker, P. J. (1964). Reproduction and heredity in trypanosomes. Int'l. Rev. Cytol. 17, 51.

Walker, P. J. and E. A. Opiyo (1973). The effect of parasite burden on the curative dose in experimental trypanosomiasis. Trans. Roy. Soc. Trop. Med. Hyg. 67, 281.

Waring, M. J. (1965). The effects of antimicrobial agents on RNA polymerase. Molec. Pharmacol. 1, 1.

Waring, M. J. (1970). Variation of the supercoils in closed circular DNA by binding of antibiotics and drugs; evidence for molecular models involving intercalation. J. Mol. Biol. 54, 247.

Webb, J. M. and H. B. Levy (1955). A sensitive method for the determination of deoxyribonucleic acid in tissues and micro-organisms. J. Biol. Chem. 213, 107.

Wijers, D. J. B. and K. C. Willett (1960). Factors that may influence the infection rate of Glossina palpalis with Trypanosoma gambiense. II. The number and morphology of the trypanosomes present in the blood of the host at the time of the infected feed. Ann. Trop. Med. Parasit. 54, 341.

Williamson, J. (1970). Review of chemotherapeutic and chemoprophylactic agents, in Mulligan, H. W. (ed.), The African Trypanosomiases. George Allen and Unwin, Ltd., London. 125.