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Clarion E. Johnson
Yale University

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CLARION E. JOHNSON

MARCH, 1976

YALE

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PENTAMIDINE TRANSPORT IN *TRYPANOSOMA BRUCEI*:
HOST SERUM PROTEIN INTERACTIONS

A Dissertation

Presented to the Faculty of the Medical School

of

Yale University

in Partial Fulfillment of the Requirements for

the Degree of Doctor of Medicine

by

Clarion E. Johnson

March, 1976

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ABSTRACT

Trypanosomes and the diseases they cause in man and animals are still important problems in Africa today. Pentamidine, an aromatic diamidine, is one of the drugs presently used in the chemotherapy and chemoprophylaxis in the trypanosomiases.

A specific transport system for the uptake of this drug by trypanosomes has been described using radioactive tracers, kinetic analysis of uptake, and cell fractionation. Drug uptake was studied by rapid centrifugation of organisms from exposing solutions through silicone into a solution of perchloric acid (PCA). This technique separates cells from extracellular drug and localizes intracellular drug in the PCA soluble fraction. Intracellular accumulation of [^{3}H]-pentamidine by _Trypanosoma brucei_ follows Michelis Menten kinetics.

Since trypanosomes live in the blood plasma of their hosts, it was important to determine whether serum proteins affect pentamidine uptake. In the present study it was determined that whole serum and immunoglobulins (IgM, IgG_1, IgG_2) inhibit pentamidine transport. Inhibition of pentamidine uptake was dependent on the concentration present in the exposing solution. Kinetic studies revealed that serum proteins do not change K_m values for pentamidine uptake. However, IgM and probably the other immunoglobulin classes reduce V_{max} values. The kinetics of uptake of pentamidine was studied in the presence of IgM. The levels of all immunoglobulins, especially IgM, increase in man and domestic animals when they are infected with African trypanosomes.

Uptake of labeled drug by trypanosomes was studied in an intact rat and a rat injected with IgM. _In vitro_ studies suggested that increased

levels of circulating IgM would reduce drug uptake and therefore reduce trypanocidal activity of pentamidine. Although in vivo studies did not necessarily support this conclusion, there is reason to speculate that pentamidine as an effective transport substrate is reduced by host immunoglobulins.

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CHAPTER I

Introduction

African sleeping sickness of man and nagana of cattle are caused by a group of closely related genetic variants of Trypanosoma (Trypanozoon) brucei Plimmer and Bradford, 1899 (Hoare, 1972). These parasites undergo striking morphological changes and antigenic variation during their life cycle. They are transmitted to mammalian hosts by several species of Glossina (tsetse flies) during the vector's blood meal. These flies are confined to tropical Africa between 15°N and 30°S. Approximately 4.5 million square miles of sub-saharan Africa, much of it with agricultural potential, are interdicted by the presence of these flies (Minter, 1972).

There are two forms of human trypanosomiases; acute (rhodesian) and chronic (gambian). Following the bite of an infected tsetse, a local reaction called a furnicle develops followed by regional lymphadenitis which lasts one to two weeks. The organisms invade the bloodstream and other tissues. In the chronic form of the disease they also invade the central nervous system (CNS) (Plorde, 1973).

CNS involvement produces the characteristic appearance of indifference, apathy, drowsiness and confusion. Focal neurological signs are usually absent, but loss of sphincter control and loss of coordination frequently occur. During this stage of the disease, intercurrent infections are common and often a major cause of death. Complications of the more acute form of the disease are serous effusion and a pericarditis. Death may occur before CNS involvement (Hutt, Wilke, 1972).

The desirable goal of complete vector eradication shows limited promise for the near future. Though the number of flies have been reduced and in

most places the disease is less feared for the moment, the flies still thrive in grasslands and forests and river courses, and the organisms they pass still kill thousands of animals and people each year. Danger yet exists (McKelvey, 1973). Therefore, chemoprophylaxis and chemotherapy are critical in the control of these diseases. Striking to the interested observer is the limited chemotherapeutic armamentarium for the treatment and or prophylaxis of trypanosome infections, and resistance has been reported against the few drugs that are available.

Drug resistance and a frequently developed cross resistance have been observed by several investigators (Yorke, 1929; Fulton and Grant, 1955; and Williamson, 1970). Chemotherapy at present is limited to drugs which lack selective toxicity for parasites over host cells (Williamson, 1962). These facts combined with a lack of knowledge about essential intracellular mechanisms leaves a scientist without knowledge as to what reactions one should aim to block (Newton, 1975). Investigation of resistance is further complicated by the striking morphological and antigenic variation particular to this organism (Gray, 1965). The role of primary intracellular and extracellular binding mechanisms and their role in resistance is yet to be elucidated (Newton, 1975). In addition, questions concerning the role of the lipoprotein structure of the cell membrane and factors influencing drug permeation need to be solved. A postulated pinocytotic mechanism of uptake for certain drugs does exist (Wang, 1965; Peters, 1974).

The use of pentamidine in the control and reduction of transmission rates of sleeping sickness is one of the best examples of practical application of long-term chemoprophylaxis (Weinman, 1968). The specific mechanism of action of pentamidine in trypanosome infections is yet to be determined. Pentamidine inhibits cell growth in <u>Escherichia coli</u> K_{12} (Amos and Vollmayer, 1957),

Staphylococcus aureus (Gale and Folkes, 1967) and *Leptomonas* (Goldberg *et al.*, 1974). It also inhibits DNA, RNA and protein synthesis in $6H_3$ HID ascites tumor cells (Bornstein and Yarbro, 1970) and *S. aureus* (Gale and Folkes, 1967). Hill and Hutner (1960) describe impaired amino acid accumulation in *S. aureus* and reduced oxygen consumption in *Crithidia fasciculata* when these organisms were exposed to pentamidine.

Recently a pentamidine transport system in *T. brucei* was characterized (Damper and Patton, 1976a; 1976b). The system is specific for the amidine moiety of the drug. It is concentrative and energy dependent. The kinetic parameters for drug transport and sensitivity are altered in trypanosomes that are resistant to diamidines. In drug resistant strains and procyclics, drug uptake is reduced *in vitro*.

Preliminary studies show that whole serum and immunoglobulins reduce the rate of drug uptake *in vitro* while serum albumin stimulates uptake (Damper, 1975). However, the effect of serum and immunoglobulins on pentamidine transport was not characterized in kinetic terms. Nevertheless, these findings are especially relevant because animals and man infected with *T. brucei* subspecies present with exceptionally high serum levels of IgM and a corresponding decreased level of serum albumin (Desowitz, 1970).

The major objective of this investigation was to determine if serum proteins affect kinetic parameters for pentamidine transport in trypanosomes.

CHAPTER II

Materials and Methods

A. Organisms:

A monomorphic, highly virulent strain of *Trypanosoma brucei* was used in these studies. This strain was derived from the pleomorphic, EATRO laboratory strain 110 after repeated syringe passages through rodents. When this study was begun, the stabilate was in its 53rd rodent passage.

B. Host Animals:

T. brucei readily infects rats. The doubling time of *T. brucei* in rats and mice is approximately 8.5 hours. Female albino Sprague Dawley rats (The Charles River Breeding Laboratory, North Wilmington, Mass.) were used as hosts. They were infected intraperitoneally (IP) and trypanosomes were isolated from their blood as described below. This strain of rats was also used as source for normal serum and for *in vivo* experiments.

C. Collection and Preparation of Trypanosomes:

Rats were bled by cardiac puncture, and the blood was defibrinated with glass beads. Trypanosomes were separated from blood cells by differential centrifugation for 15 minutes at 5° at 1000 x g (swinging bucket, PRI International centrifuge). The serum and buffy coat containing the parasites were removed and pipetted into another centrifuge tube. Rabbit anti-rat blood cell antibody was added to this cell suspension, and incubated for 15 minutes, at room temperature. The suspension of organisms was then repeatedly centrifuged at 1000 x g, as above, in order to separate contaminating blood cells and platelets. Contamination was determined by microscopic examination. Except for the 15 minute period when rabbit and anti-rat blood cell antibody was added, the trypanosomes were kept cold and in host serum throughout the

isolation procedure. Ten µl of the suspension were diluted in a unopipette blood diluting pipette (Becton and Dickinson) and the parasites were counted in the improved Neubauer hemocytometer. An aliquot of the parasite suspension was centrifuged for one minute at 12,000 x g at 5° in a Sorvall Superspeed RC2B centrifuge immediately before being used in uptake studies. The supernatant was discarded and the trypanosomes were resuspended in Hanks' balanced salt solution containing 100 mg% (w/v) glucose (HBSS) and 50 mg% (w/v) albumin (HBSSA) so that the concentration of the trypanosomes was 2 x 10^8 per ml. Where appropriate, trypanosomes were resuspended in HBSS containing whole serum or serum fractions.

D. Serum Fractionation:

All serum used in experiments was collected from rats with no history of trypanosomiasis. Serum was chromatographed on a Sephadex G-200 ascending column: bed volume = 350 ml; eluant was HBSS without glucose, pH = 7.3. Each fraction was monitored at 280 nm in a Gilford 2400 Spectrophotometer. Fractions for each peak were pooled and concentrated in an Amicon ultrafiltration apparatus under 60 psi nitrogen, using a Diaflo PM10 ultrafiltration membrane. The IgM, IgG_1, IgG_2, and albumin enriched fractions were used as additions in transport experiments.

E. Protein Determinations:

Protein determinations were made using the method described by Lowry, et al. (1951).

F. Transport Studies:

All experiments were carried out at 37°. Solutions of HBSS containing two times the final concentration of [^{3}H]-pentamidine were thermally equilibrated in 37° H_2O bath. A volume of washed trypanosomes (2 x 10^8 cells per ml) was pipetted into prewarmed 50 ml erlenmeyer flasks each of which

contained a 15 mm stirring bar. The flask containing the organisms was then incubated in a water bath for 45 secs. A volume of [^{3}H]-pentamidine solution equal to the volume of cell suspension was pipetted into the flasks and immediately mixed on a magnetic stirrer. An electric timer (Lab Chron Timer, Labline Instruments, Inc.) was activated at the time [^{3}H]-pentamidine was added.

At intervals of 10-15 seconds the contents of the flask were mixed and 250 µl of the suspension were removed with an Eppendorf Automatic pipette, and layered on top of 50 µl of silicone (G.E. versilube F 50; viscosity = 75 centistokes, specific gravity = 1.05) which was layered over 100 µl of 12% (v/v) perchloric acid (PCA) all contained in a 500 µl plastic microfuge tube (See Fig. 1 in appendix). The specific gravity of the PCA was approximately 1.1. Organisms were then spun from the radiolabeled exposing solution through the discontinuous gradient into the PCA layer by centrifugation for 1 minute at 7,000 g in a Beckman 152 microfuge. When the cells reach the PCA, they are lysed, their soluble contents are released, and the PCA precipitated material is pelleted.

Ten µl of the cell free solution were taken from the layer above the silicone for radioactivity determination. Then the microfuge tube was cut with a scalpel below the silicone layer and 50 µl of the PCA soluble material was taken from the PCA layer. These samples were placed in 4 dram vials and scintanalyzed in 4 ml of counting solution. The solution consisted of 1 gram bis-MSB [para-bis-(O-methylstyryl)-benzene] and 7 gram PPO (2,5, diphenyl oxazole) dissolved in 340 ml of Triton X-100 and 600 ml of xylene. Samples were counted in a Beckman LS 250 liquid scintillation counter.

G. Chemical Compound:

The [^{3}H]-pentamidine used in this experiment was prepared from the free

base by New England Nuclear. The isolation, purification, and verification of chemical purity have been described previously (Damper and Patton, 1976a).

CHAPTER III

Results

Part A. Characterization of [^{3}H]-pentamidine transport by *T. brucei* in the presence of rat serum and serum fractions

1. [^{3}H]-pentamidine uptake in the presence of whole rat serum

In the following experiment whole serum was added to the transport solution in order to determine if serum modifies [^{3}H]-pentamidine transport in *T. brucei*. The external concentration of pentamidine in this experiment was 24 μM. As shown in Fig. 1 and Table I, as the concentration of serum is increased from 4 mg to 40 mg/ml, the rate of pentamidine transport by *T. brucei* is decreased (Fig. 1A–E). For each increase of 10 mg/ml of serum protein in the transport vessel, the rate decreases by 41.5 pmoles pentamidine/min/1.25 x 10^7 trypanosomes (Fig. 1F).

2. [^{3}H]-pentamidine uptake by *T. brucei* in the presence of various serum fractions

Rat serum was chromatographed as described in Materials and Methods. Figure 2 shows the optical density of fractions read at 280 nm. The peaks represented on the graph correspond with the IgM, IgG_1, IgG_2 and albumin fractions of rat serum.

The most enriched fractions representing each serum protein were combined and concentrated by ultrafiltration as described in Materials and Methods. In experiments 125 mg/ml of IgM, IgG_1 and IgG_2 reduced pentamidine uptake in *T. brucei*, but kinetic constants were not determined for uptake in the presence of IgG. Drug uptake was most reduced in the presence of IgG_2 and least reduced in the presence of albumin (Fig. 3 and Table II).

3. Kinetics of [^{3}H]-pentamidine uptake in the presence of albumin and IgM

The effect of IgM on pentamidine transport was studied in more detail

Fig. 1. Initial rates of pentamidine uptake by *T. brucei* in the presence of various concentrations of whole serum. Control (A), 4 mg protein/ml (B), 15 mg protein/ml (C), 24 mg protein/ml (D), 40 mg protein/ml (E). Figure 1.F represents rates of uptake from the above data plotted against mg serum protein in solution.

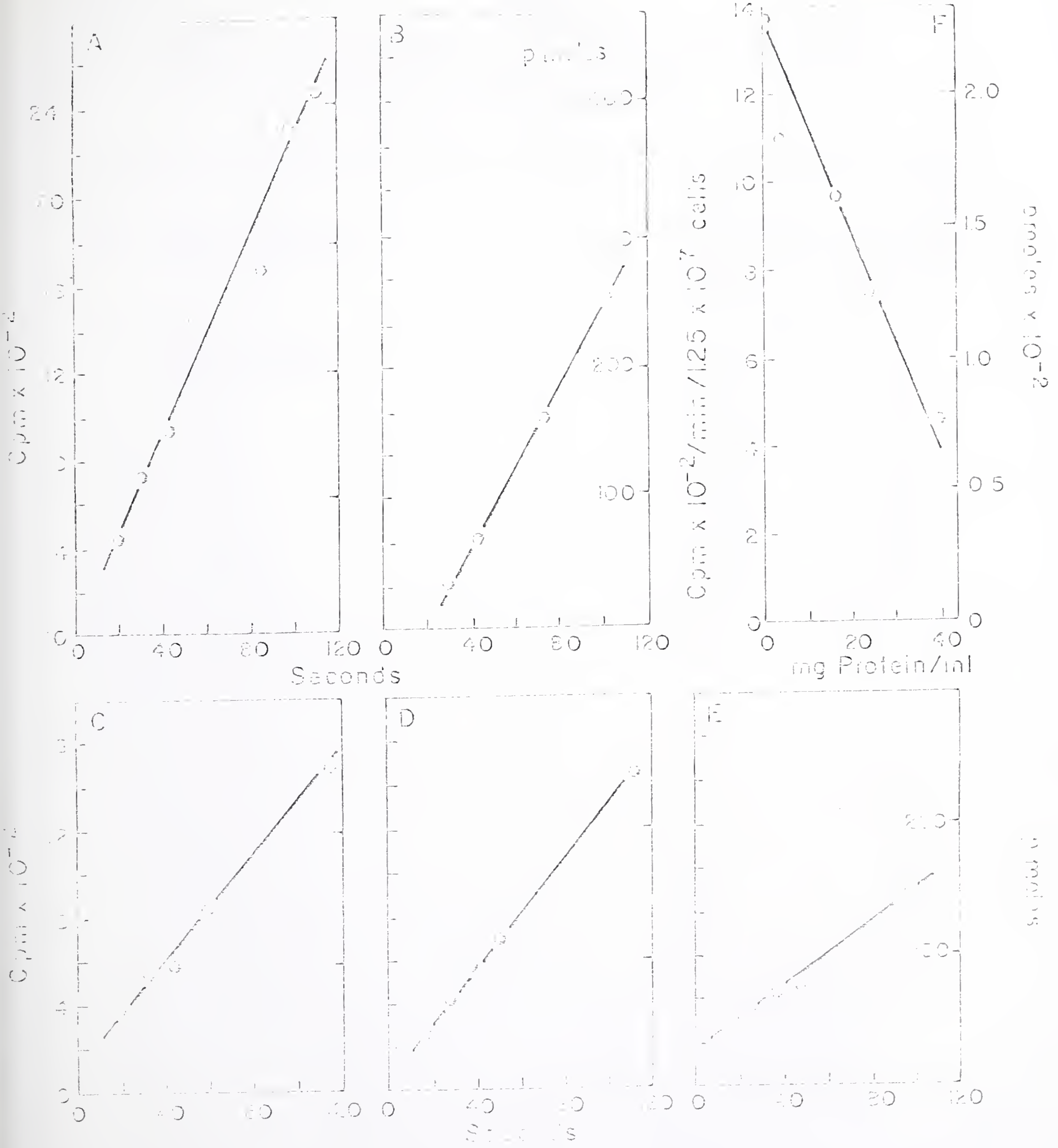
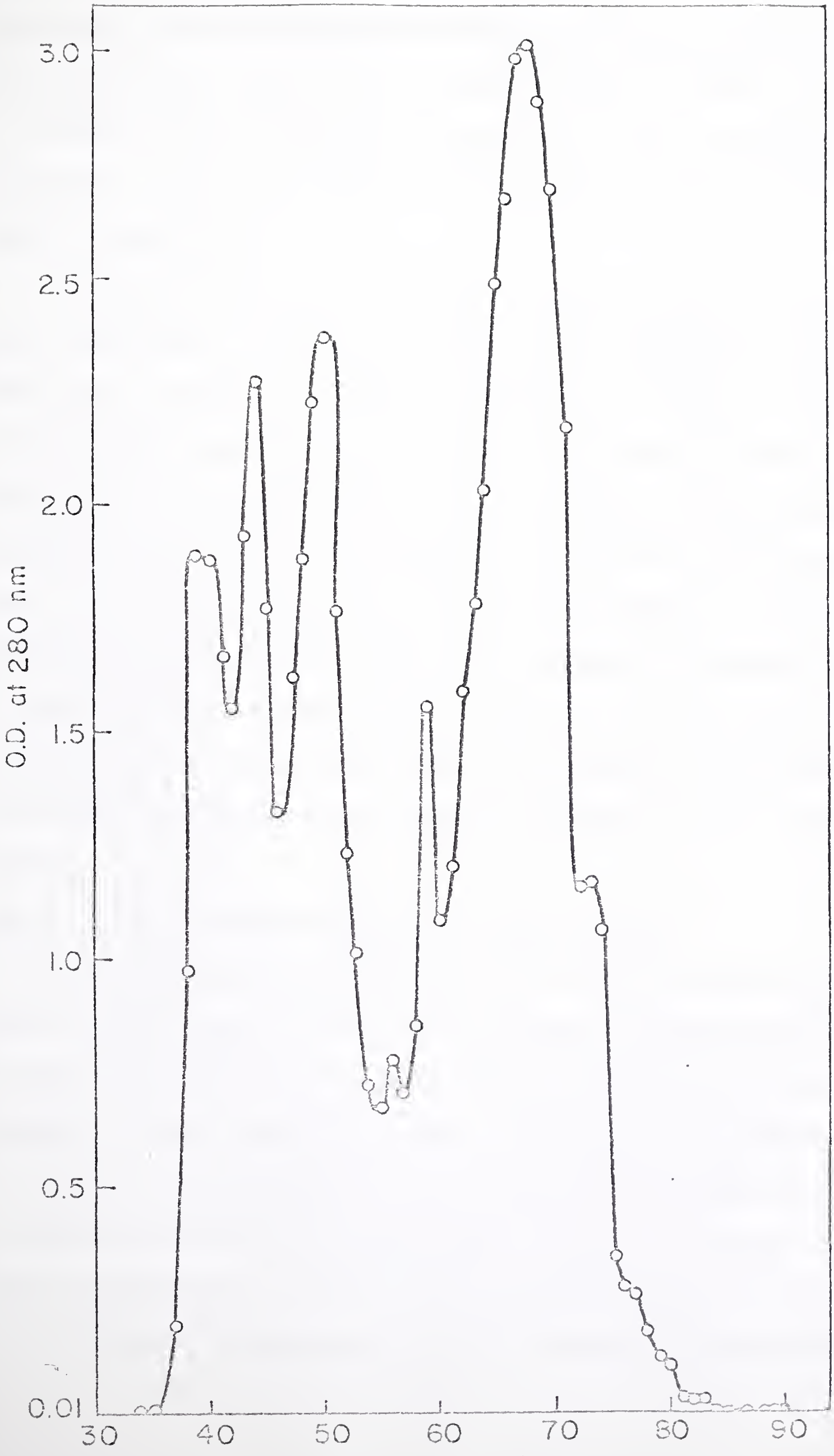

A
B
C
D
E
F
Cpm x 10^{-2}
Cpm x 10^{-2}/min/1.25 x 10^{7} cells
pmoles x 10^{-2}
pmols
pmols
Seconds
Seconds
mg Protein/ml
0
4
8
12
16
20
24
100
200
300
400
0
2
4
6
8
10
12
14
0
0.5
1.0
1.5
2.0
100
200
0
40
80
120
0
20
40

Table I

Effect of Various Serum Concentrations on Initial rate of Uptake in 3.5 μM [^{3}H]-pentamidine

Final serum protein concentration (mg protein/ml)	Initial rate of uptake by 1.25 x 10^7 trypanosomes cpm/min	pmoles/min
Control (HBSSA)	1360	225
4	1100	185
15	960	160
24	750	125
40	460	75

Fig. 2. Chromatography of normal rat serum on an ascending Sephadex G-200 column. Three ml fractions were collected. Protein content was detected from light absorption at 280 nm. The IgM enriched fraction was eluted in the void column and is indicated by the first peak. IgG_1 is indicated by the second peak and IgG_2 by the third peak. The smaller peaks were not used in experiments. The last and highest peak represents albumin. Fractions 37 through 40 (IgM); 45 through 47 (IgG_1); 48 through 53 (IgG_2); and 63 through 72 (albumin) were used in transport studies.


O.D. at 280 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.01
30
40
50
60
70
80
90
Fraction number

because this immunoglobulin class is exceptionally elevated in trypanosome infections. In the following experiments K_m and V_{max} values were determined for pentamidine transport in the presence of IgM or albumin in order to see if inhibition of drug uptake in the presence of IgM was competitive or non-competitive. All rate constants were derived from initial rates of pentamidine uptake (Fig. 4).

All rates of drug uptake in various concentrations of [^{3}H]-pentamidine were reduced when 125 mg/ml of IgM were in the exposing solution (Fig. 5, Table III). As shown on a double reciprocal plot in Fig. 6, the intercept on the 1/[Pent] axis is the same. Since the intercept is equal to $-1/K_m$, the K_m value for uptake is the same in the presence of albumin and IgM, but the V_{max} value is lower in the presence of IgM than in the presence of albumin. In experiments repeated 3 times using 3 different trypanosome populations, K_m values differed (Table III). However, it should be noted that K_m values are the same within a given experiment in the presence of IgM. On the other hand, the V_{max} value within an experiment is consistently lower in the presence of IgM than in the presence of albumin. Values were reduced by 30-65%.

Part B. [^{3}H]-pentamidine uptake in T. brucei in vivo

The preceding experiments examined the effects of serum and serum fractions on drug transport in vitro. The following experiments were designed to study interaction between drug uptake in trypanosomes and plasma proteins in vivo. All blood samples were taken up in heparinized capillary tubes. The uptake of [^{3}H]-pentamidine by rat blood cells, peritoneal exudate cells, and hepatic cells has been studied (Damper, 1975). The results show that uptake is concentration dependent and that the rate for uptake by exudate cells and hepatic cells is much less than is observed for the parasites.

Fig. 3. Initial rates of [^{3}H]-pentamidine uptake by *T. brucei* in the presence of serum fractions enriched for IgM, IgG$_1$, IgG$_2$ and albumin.

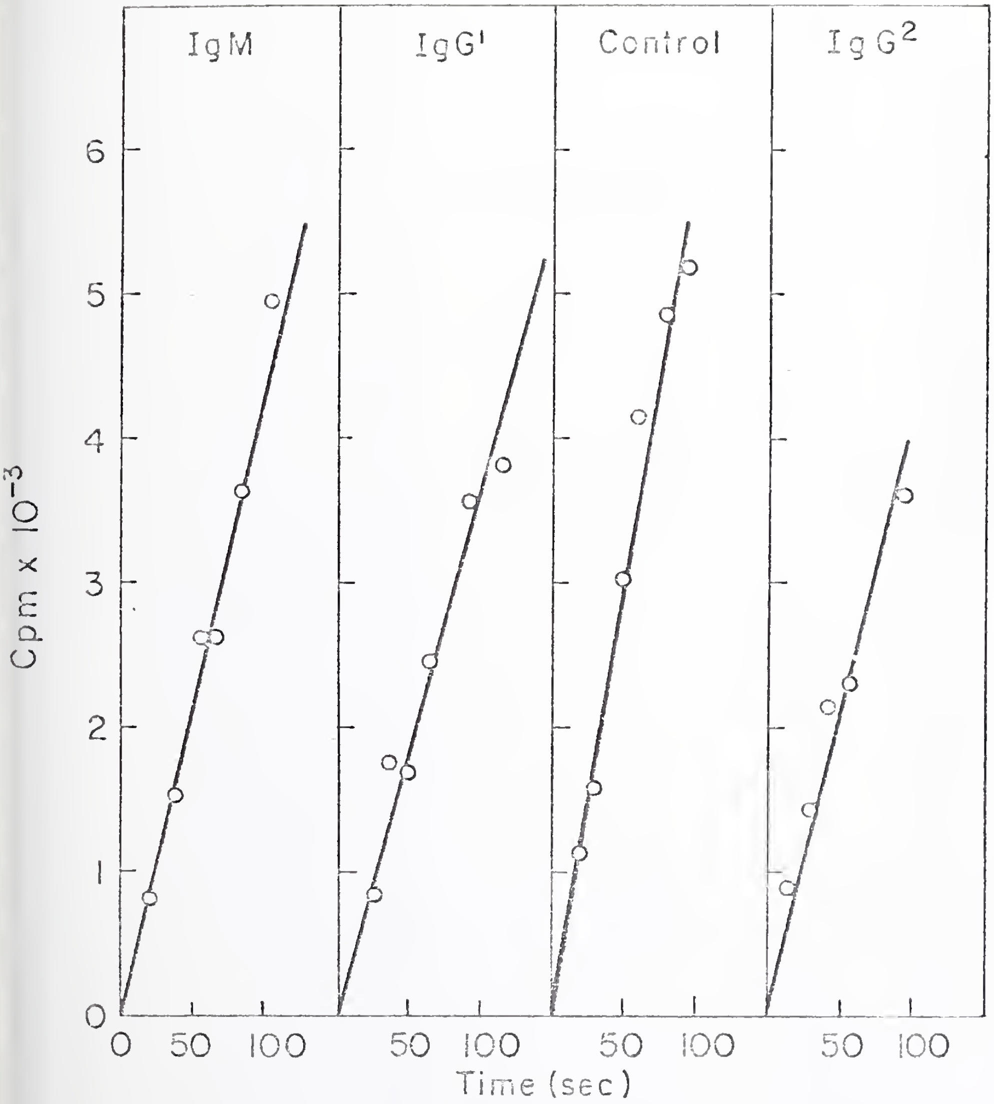

IgM
IgG¹
Control
IgG²
Cpm x 10⁻³
0
1
2
3
4
5
6
0
50
100
50
100
50
100
50
100
Time (sec)

Table II

Initial Rates of [^{3}H]-pentamidine Uptake

by *T. brucei* in the Presence of Various Serum Fractions

	pmoles pentamidine taken up/min by 1.25 x 10^7 trypanosomes		Percent of control	
External pentamidine	24 μM	6 μM	24 μM	6 μM
Serum Fraction				
Control (HBSSA)	336.0	69.3	100	100
IgM	232.6	59.4	70	85.7
IgG_1	-----	37.4	---	53.4
IgG_2	144.0	21.2	42	30.6

Fig. 4. Initial rates of [^{3}H]-pentamidine uptake by *T. brucei* at 12 μM (a), 6 μM (b) and 3 μM (c) drug in the presence (● ▲ ■) and absence (○ △ □) of IgM (125 mg protein/ml).

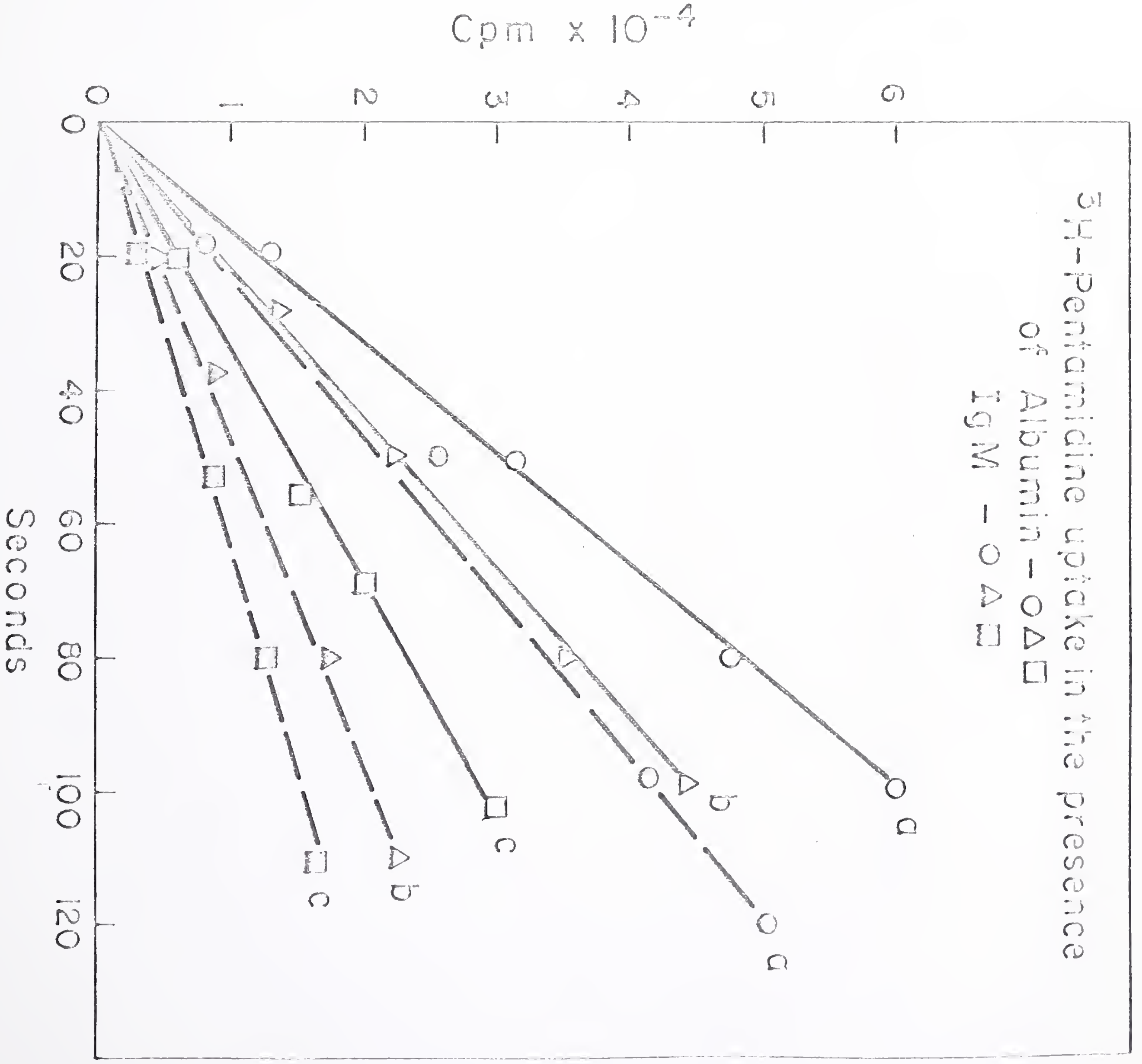
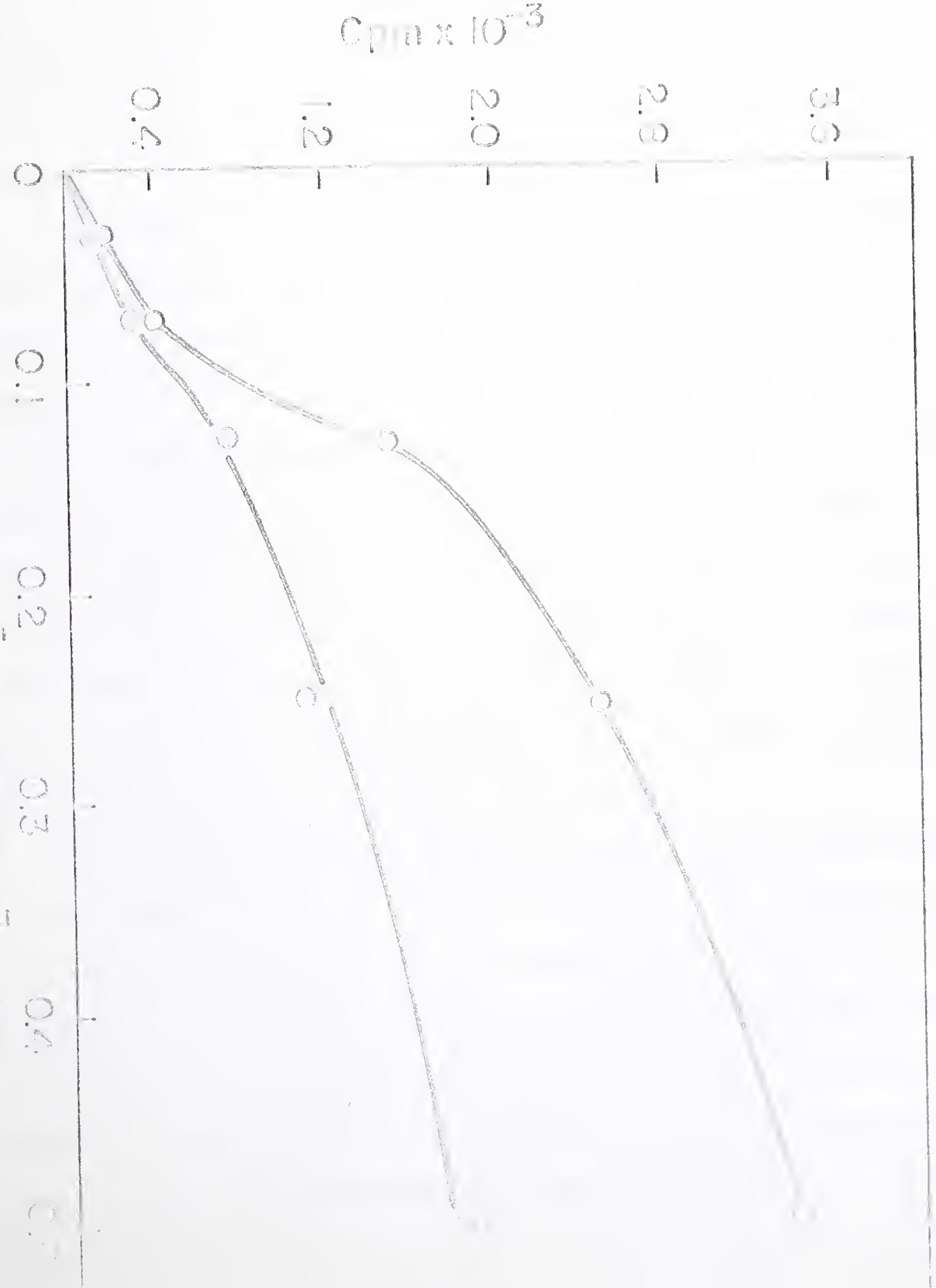

^{3}H-Pentamidine uptake in the presence
of Albumin – ○△□
IgM – ○△□
Cpm x 10^{-4}
0
1
2
3
4
5
6
Seconds
0
20
40
60
80
100
120
a
b
c
a
b
c

Fig. 5. A plot of rates vs pentamidine concentration in the presence (●) and absence (0) of IgM (125 mg protein/ml). Final drug concentrations were 0.061 μM, 0.125 μM, .250 μM, and 0.5 μM.


Cpm x 10^{-3}
0.4
1.2
2.0
2.8
3.6
0
0.1
0.2
0.3
0.4

Erythrocytes exclude the drug, thus the PCA soluble $[^3H]$-pentamidine in the *in vivo* studies probably represents $[^3H]$-pentamidine taken up by trypanosomes. Little or no PCA soluble radioactivity is found in the absence of trypanosomes.

1. $[^3H]$-pentamidine uptake by T. brucei in an intact rat

A curative dose of $[^3H]$-pentamidine, 1 mg/kg, was injected I.P. into a 215 g rat with a parasitemia of 7.0×10^5 trypanosomes/cmm. Immediately following this, samples of tail blood were monitored over a period of 20 hrs for red blood cell (RBC) count, parasitemia, and intra- and extra-cellular radioactivity.

Fig. 7 shows that the RBC count fluctuates between 12.25×10^6/cmm and 6.4×10^6/cmm. These fluctuations do not appear to form a trend, and may be due to periodic variations in the number of erythrocytes in peripheral circulation and observer error. Trypanosome counts also fluctuated but with a downward trend. Within minutes following injection of $[^3H]$-pentamidine, radioactivity was found in the blood plasma. After an initial rise, the level of radioactivity remained at approximately 156 cpm/0.01 μl of whole plasma. Total intracellular radioactivity rose and roughly corresponded with a precipitous drop in parasitemia by 5 hrs after introducing the drug. Five hours after the drug was injected, the highest intracellular radioactivity was 1.6×10^3 cpm/10^5 trypanosomes.

2. $[^3H]$-pentamidine uptake by T. brucei in vivo in rat injected with IgM

IgM inhibited uptake of pentamidine *in vitro*. The following experiment examines *in vivo* drug accumulation by *T. brucei* in rats injected with IgM. A 200 g rat with a parasitemia of 10^6 organisms/cmm was injected I.P. with 1 ml of HBSS containing 125 mg of IgM. Immediately following this, a curative dose of $[^3H]$-pentamidine was injected after which, samples of tail blood were taken over a period of 11 hours.

Fig. 6. A comparison of double reciprocal plots of initial rate of pentamidine uptake at various concentrations in the presence of 1 ml of albumin (●) and 125 mg protein/ml of IgM (▲).

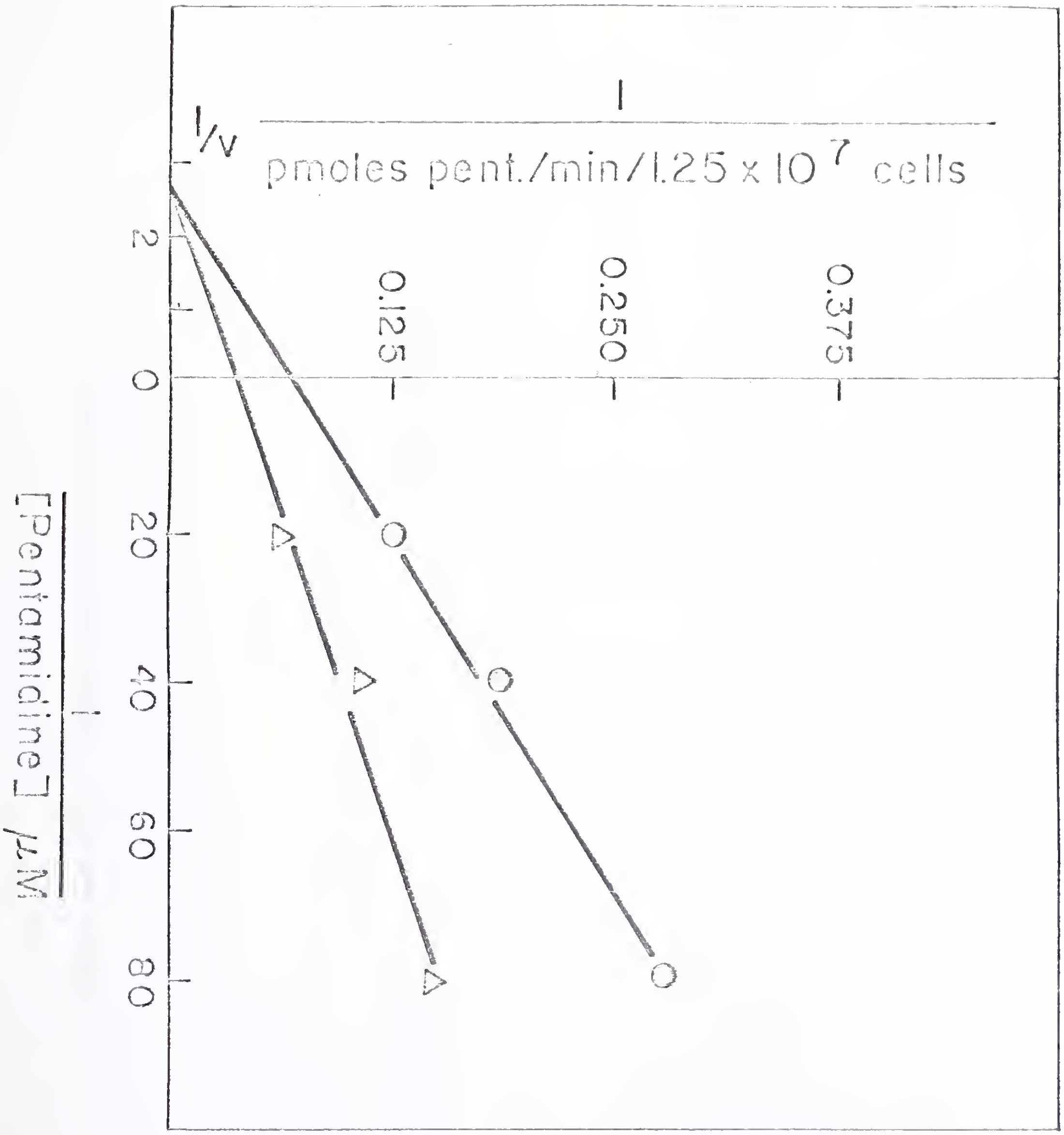
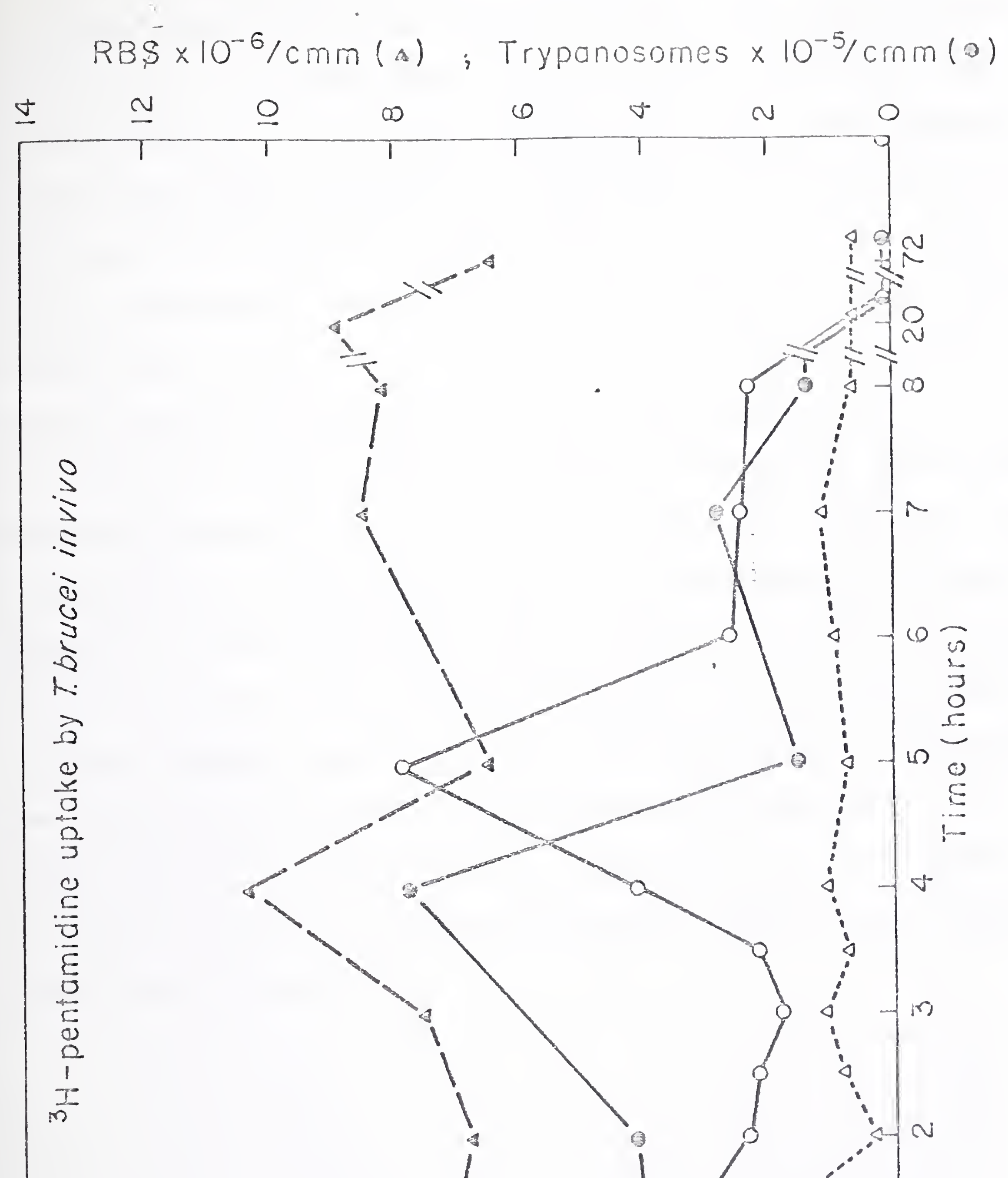

1/v
1
pmoles pent./min/1.25 x 10^7 cells
0.125
0.250
0.375
0
2
20
40
60
80
1
[Pentamidine] μM

Table III

Kinetic Constants for Pentamidine Transport in the Presence of Albumin and IgM

Albumin (Control)		IgM		V_{max}	
K_m (µM)	$V_{max}(\frac{\text{pmoles/min}}{1.25 \times 10^7 \text{ cells}})$	K_m (µM)	$V_{max}(\frac{\text{pmoles/min}}{1.25 \times 10^7 \text{ cells}})$	% Control rate	% Reduced in IgM
10.00	27.80	6.67	10.4	37	63
2.56	71.4	2.56	50.00	70	30
2.78	28.5	2.78	10.00	35	65

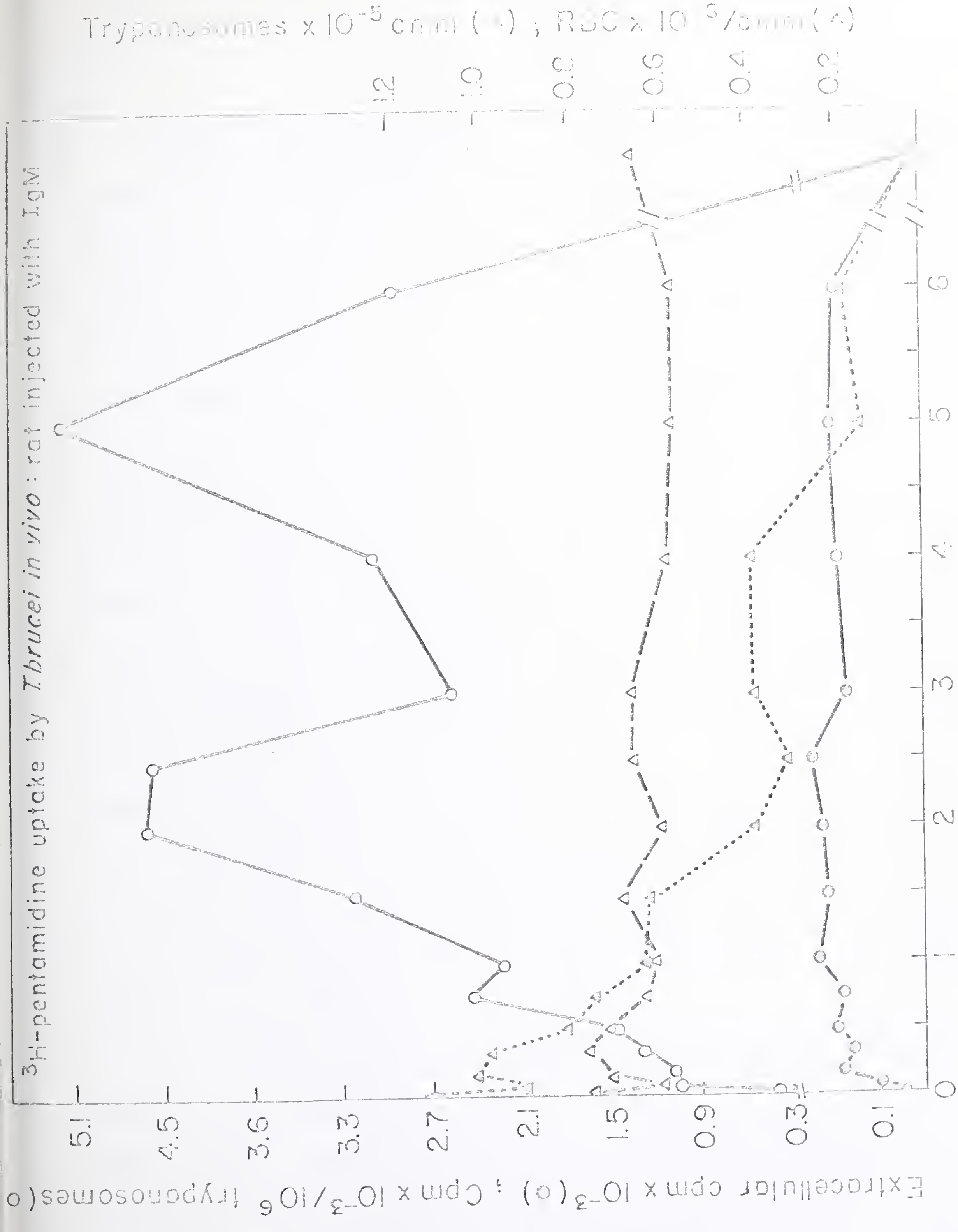
Fig. 7. Trypanosome levels (●), RBC count (▲), plasma associated radioactivity (△) and cell associated radioactivity (○) in a rat infected with <u>T.</u> <u>brucei</u> and injected IP with 1 mg of $[^3H]$-pentamidine/Kg body wt.



^{3}H-pentamidine uptake by *T. brucei in vivo*

Fig. 8 shows fluctuations in red cell number. There doesn't appear to be any trend. The parasite number decreased from 1.1 x 10^6/cmm to zero in 11 hours within 1 hr after [^{3}H]-pentamidine was injected. Extracellular radioactivity rapidly increased and leveled off at about 200 cpm/0.01 μl. This level of radioactivity was maintained for at least 6 hours. Intracellular radioactivity rose to more than 4.5 x 10^3 cpm per million trypanosomes within 2 hrs after the tritiated drug was injected. Parasitemia dropped during this period from 1.1 x 10^6 trypanosomes/cmm down to 4 x 10^5/cmm. Three hours after the drug was injected, intracellular cpm decreased and then increased for the next 2 hours to a peak of 5.1 x 10^3 cpm. After the intracellular radioactivity dropped, parasitemia leveled off at approximately 4 x 10^5/cmm and then decreased to less than 2 x 10^5/cmm. The decrease in parasitemia was associated with a rise in intracellular radioactivity. Between 6 and 11 hours after introducing the drug, trypanosomes and intracellular radioactivity disappeared from the bloodstream. The highest intracellular radioactivity per trypanosome in the rat injected with IgM was about 1/3 the highest intracellular radioactivity of trypanosomes from the untreated rat.

Fig. 8. Trypanosome levels (▲) RBC counts (△), plasma associated radioactivity (●) and cell associated radioactivity (○) in a rat infected with _T. brucei_ and injected IP with 125 mg of IgM and a curative dose of pentamidine.


3H-pentamidine uptake by T.brucei in vivo : rat injected with IgM
Trypanosomes x 10⁻⁵ cmm (•) ; RBC x 10⁻⁵/cmm (△)
1.2
1.0
0.8
0.6
0.4
0.2
Extracellular cpm x 10⁻³ (o) ; Cpm x 10⁻³/10⁶ trypanosomes (o)
5.1
4.5
3.6
3.3
2.7
2.1
1.5
0.9
0.3
0.1
Time (hours)
0
1
2
3
4
5
6

CHAPTER IV

Discussion

Transport studies, especially those involving parasites and drugs, are complex. First of all, the studies necessitate removing the organisms from the host; in the present study trypanosomes were isolated free of all host cells and platelets. In addition, the transport substrate is cytocidal. Consequently, specific safeguards are essential. The safeguards used in these studies have been presented elsewhere (Damper and Patton, 1976a). They include tests for chemical purity of the [^{3}H]-pentamidine used. To avoid artifacts due to cell death, the drug concentrations used for transport were below those concentrations which are immediately trypanocidal. No detectable adverse effects by these concentrations were observed within the time periods that the organisms were studied. Trypanosomes were examined in each experiment by light microscopy for motility, immediately before incubation in the drug and after uptake studies. When other physiological parameters were investigated, such as glucose and oxygen consumption, external concentrations of pentamidine as high as 24 μM had no effect during a period of 1 hour (Damper, 1975). Therefore, the external concentrations of drug used for transport studies do not measurably affect the general viability of the parasites.

The silicone sandwich technique for measuring uptake rapidly and quantitatively separates cells from the exposing solutions (Patton and Balber, 1976). When intracellular and extracellular [^{3}H] compound was monitored and analyzed using this technique of separation, the chemico-physical properties of the recovered tritiated compound were identical with pentamidine (Damper, 1975). Therefore, the drug is not metabolized and intracellular radioactivity represents [^{3}H]-pentamidine during the time

period studied.

Recent studies show that pentamidine is rapidly taken up by a substrate specific transport system. Proteins such as bovine serum albumin (BSA) fraction V enhance viability of trypanosomes. Thus when BSA is in the exposing solution, uptake rates are higher and more consistent. The aim of the present study was to determine if serum immunoglobulins alter pentamidine transport in _T. brucei_. Though protein free serum components had no effect on pentamidine uptake, whole serum inhibited the process (Damper, 1975). Inhibition of [^{3}H]-pentamidine uptake is dependent on the concentration of serum (Table I and Fig. 1). For each 10 mg/ml increase of whole serum in exposing solutions, there was a decrease of 41.5 pmole/min in the rate of [^{3}H]-pentamidine uptake. This demonstrates serum inhibition of uptake but does not indicate how inhibition is brought about. The two most likely explanations for this observation are: (1) whole serum interferes by binding to the transport site or (2) serum interferes by binding the drug. It is well documented that serum proteins are closely associated with surface proteins of trypanosomes (Desowitz, 1970; Vickerman, 1974). This combination of serum proteins might somehow alter membrane architecture and change membrane permeability characteristics, but this has not been demonstrated. It is more likely that serum binds [^{3}H]-pentamidine and in this way reduces the effective, transportable concentration of pentamidine. In order to determine which serum proteins affect the kinetics of pentamidine uptake, IgM, IgG$_1$, IgG$_2$ and albumin enriched serum fractions were added to transport solutions (Fig. 3, Table II). The results showed that all of the immunoglobulins reduced [^{3}H]-pentamidine uptake (Table II). Although IgG$_2$ gave the greatest inhibition, the effect of IgM on drug uptake was studied in more detail because of its clinical importance in

African trypanosomiasis.

These results are consistent with a model in which the rate of accumulation of intracellular radioactive pentamidine is limited by a single saturable component in the plasma membrane of the parasite. When other diamidines, such as stilbamidine or propamidine, are used as competitive inhibitors of pentamidine uptake, characteristically apparent K_m values for pentamidine uptake increase without a change in V_{max} values. The observation argues for structural specificity in regards to pentamidine affinity for the transport site (Damper and Patton, 1976a). IgM does not change the K_m value for pentamidine transport and this suggests that affinity between pentamidine and the saturable component is not altered by the addition of IgM (Table III and Fig. 6). Therefore, IgM does not compete for some site in the membrane used for the translocation of pentamidine.

Nevertheless, consistently within experiments, initial rates of $[^3H]$-pentamidine transport were reduced in the presence of serum and all of the immunoglobulins. Double reciprocal plots of uptake data show that V_{max} values are consistently lower in the presence of IgM than in HBSSA (Table III). It should be noted that V_{max} values varied between experiments. This variation may be explained by, (a) a selective process among antigenic variants giving rise to differences in drug response simultaneously with antibody resistance in the absence of previous contact with drugs (Cartrell, 1958; Gray, 1966; Soltys, 1957, 1959); (b) effects due to parasitemia level at the time the parasites were harvested from the rat; and (c) effects that are related to the number of days postinfection before the trypanosomes were isolated (Goldstein and Patton, 1976). None of these variables are controlled for in the present study.

V_{max} values within experiments are reduced by 30-65% in the presence of

IgM (Table III). Double reciprocal plots indicate that inhibition is of the non-competitive type. In the absence and presence of various concentrations of inhibiting substances, the plots differ in slope but do not share a common intercept on the 1/v axis. The intercept on the 1/v axis is greater in the presence of IgM than in its absence indicating that V_{max} is decreased in the presence of IgM and is not restored by high substrate concentrations (Fig. 6). The most common kind of noncompetitive inhibition is that observed when reagents combine reversibly with -SH groups of cystein residues. Such residues are thought to be essential for a variety of catalytic activities including pentamidine transport in trypanosomes (Patton and Damper, 1976a). However, there is no evidence that blood serum proteins react with -SH groups of cystein residues in such a manner.

It should be pointed out, however, that pentamidine is a potent inhibitor of the streptokinase (SK), dependent activities of human plasminogen and of the activation of bovine plasminogen by the SK-human plasmin activated complex (Geratz, 1973). Serum and immunoglobulins reduce the inhibition process. In the fibrinolytic assay of plasmin, the modifying effect of serum proteins is thought to be based on a time dependent interaction with the enzyme which presumably alters its susceptibility to inhibition by pentamidine (Geratz, 1973). This interpretation of plasma-pentamidine-plasminogen-interaction does not help explain immunoglobulin inhibition of pentamidine transport in T. brucei. Other studies indicate that pentamidine binds to immunoglobulins (see Appendix Fig. 2, Patton, unpublished data).

In that study HBSS solutions containing whole serum or serum fractions were placed in dialysis tubes. These tubes were suspended in [^{3}H]-pentamidine and the rate of radioactivity accumulation in the tubes were monitored. Both the rate and the amount of [^{3}H]-pentamidine accumulated in the tubes

were directly proportional to the concentration of serum protein in the tubes. Accumulation of [^{3}H]-pentamidine was more rapid in tubes containing IgM, IgG$_1$, IgG$_2$, than in tubes containing albumin (Appendix Fig. 2). The concentration of [^{3}H]-pentamidine within tubes which contained HBSS and albumin did not exceed the external concentration of radioactive drug during 23 hrs. Dialysis tubes which contained whole serum or immunoglobulins all accumulated concentrations which exceeded external concentrations of [^{3}H]-pentamidine. These data support the conclusion that serum immunoglobulins bind [^{3}H]-pentamidine. If this conclusion is valid, then the effective or unbound [pent]$_e$ is reduced in the presence of serum proteins, and drug uptake by trypanosomes, which depends on external drug concentrations, is thereby reduced. This leads to reduced trypanocidal activity of the drug for _T. brucei_ in the presence of serum proteins. As shown in Figure 3 in the Appendix (Patton, unpublished data), organisms in the presence of a trypanocidal concentration of pentamidine (200 μM) are killed at a rate of 10^5 cells/min if suspended in HBSSA. Similar rates of cell death are observed when up to 5 mg of serum protein are in the solution. However, as serum concentrations are increased up to 40 mg/ml, there is a corresponding decrease in cell death. Damper and Patton (1976b) have reported that the trypanocidal effect of pentamidine depends upon the parasite's ability to transport the drug. In that study, strains of trypanosomes with varying drug sensitivities were used. They found that the intracellular concentration of pentamidine at crisis in dose response studies of _T. brucei_ was approximately 1.4 μM and the time it takes organisms to concentrate pentamidine to 1.4 μM is dependent on the external concentration of the drug and the rate of uptake. The ability to transport the drug is directly related to drug effectiveness. In the present study one strain of _T. brucei_ was used, and its ability to transport the drug

is apparently inhibited in the presence of serum immunoglobulins which were not specific for trypanosomes. All of these findings may have clinical implications and be specially related to field observation of drug resistant infections. Elevated IgM is an early feature of Brucei-subgroup trypanosome infections. In human trypanosomiasis the IgM level may rise to four times the normal level (Desowitz, 1970). This elevated level of IgM is detectable in some patients before the onset of symptomatic disease. Following chemotherapy or spontaneous remission, IgM levels return to normal (Matter *et al.*, 1961). In general, sera from man and animals infected with African trypanosomes show a relative decline in albumin and an increase in gamma globulin (Desowitz, 1970). In the human and primate infection with *T. gambiense* there is an uncharacterized extra component between the beta and gamma globulins (Gall, 1956). In contrast, the fulminating disease in rodents infected with African trypanosomes is characterized by lower gamma globulins and alpha globulin levels. These animals show almost no resistance to *T. brucei*. Therefore, rats were used in an attempt to confirm and better interpret the results obtained *in vitro* with pentamidine and blood serum proteins. These studies were not, in the strictest sense, transport studies but rather they were studies to see if accumulation of pentamidine by *T. brucei* is altered by host serum proteins *in vivo*.

Since circulating serum proteins were not measured and so few animals were used, the studies are not definitive and are difficult to interpret. They do suggest that such studies in rodents are possible and that questions about host interactions in the chemotherapy of trypanosomiasis using pentamidine can be asked. The study shows that intracellular radioactive drug can be monitored. In the *in vivo* experiments, intracellular drug concentration increased with time until a critical concentration was reached; at which time

parasitemia levels dropped precipitously (Figs. 7 and 8). It was hoped that these preliminary studies would show that increased levels of circulating immunoglobulins prolonged the period between treatment and elimination of parasites. Such results would be predicted from the data obtained from in vitro studies. However, the data from 2 experiments are too few. Certainly further studies are indicated before any conclusions can be drawn concerning interactions between drug and host serum proteins and trypanosomes in vivo.

Transport parameters for [^{3}H]-pentamidine have been examined for specifics of inhibition of uptake by serum proteins. Evidence is presented that [^{3}H]-pentamidine transport is inhibited in the presence of whole serum. The kinetics of pentamidine transport in IgM containing solutions show that inhibition is noncompetitive. Since effectiveness of pentamidine as a trypanocidal agent is dependent first of all on its ability to permeate the cell membrane, serum immunoglobulins by reducing the rate of drug uptake, inhibit the trypanocidal action of pentamidine in vitro. In vitro experiments might suggest that similar interactions occur in the host. Pentamidine is sequestered by host tissues (Fulton and Mathew, 1959; Launoy and Jonchere, 1960), and there is cause to speculate that it is reduced as an effective transport substrate by host immunoglobulins. This situation is very likely enhanced in trypanosome infections in man and animals where increased levels of immunoglobulins and decreased levels of albumin are observed.

APPENDIX

Discontinuous gradient system, and studies on the binding of [^3H]-pentamidine to serum proteins and the sparing effect of serum on the trypanocidal activity of pentamidine.

Fig. 1. Schematic representation of the microfuge tubes used in transport studies before and after centrifugation. (After Patton, C. and Balber, A. 1976. J. Protozoology, in press.)

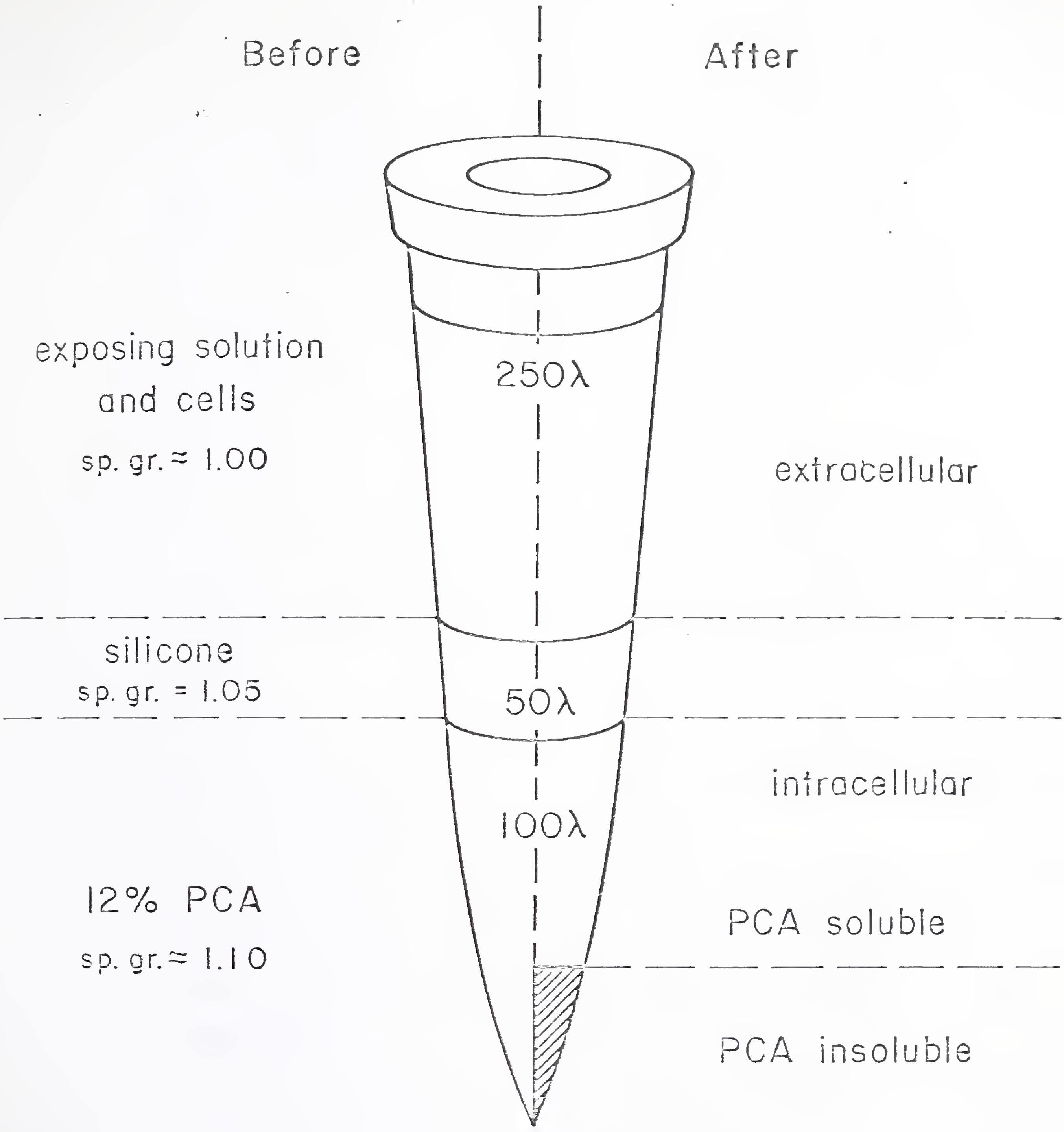

Before
After
exposing solution
and cells
sp. gr. ≈ 1.00
250λ
extracellular
silicone
sp. gr. = 1.05
50λ
intracellular
100λ
12% PCA
sp. gr. ≈ 1.10
PCA soluble
PCA insoluble

Fig. 2. Accumulation of [^{3}H]-pentamidine (Sp. Ac. = 100 dpm/pmole) in dialysis tubes containing 1 ml of various concentrations of whole serum and serum fractions. For assaying accumulation 1 ml of serum solution or fraction was pipetted into 10 cm long tubes sealed at one end with a polyethylene stopper and gasket. Tubes had been previously boiled in 1.0% (w/v) Na_2CO_3, double distilled water, 0.001M EDTA, and finally, double distilled water. Each tube containing serum or serum fractions was then suspended from a Lucite holder which was in turn attached to a motor that rotated the holder 180° clockwise and then 180° counterclockwise alternately. The tubes containing the samples were suspended in 0.526 μmolar [^{3}H]-pentamidine (Sp. Ac. = 100 dpm/pmole) in the cold (5°).

A. Radioactivity at various time intervals in 50 μl of fluid in tubes containing 40 mg (□), 20 mg (△), 10 mg (■), 5 mg (●), 2.5 mg and 0 mg (▲,○) of whole serum in HBSS.

B. Radioactivity at various time intervals in 50 μl of fluid in tubes containing HBSSA (○), 3 mg of serum fractions enriched for IgM (□), IgG$_1$ (△), IgG$_2$ (▲) and albumin (   ) (Patton, C.L., unpublished data).

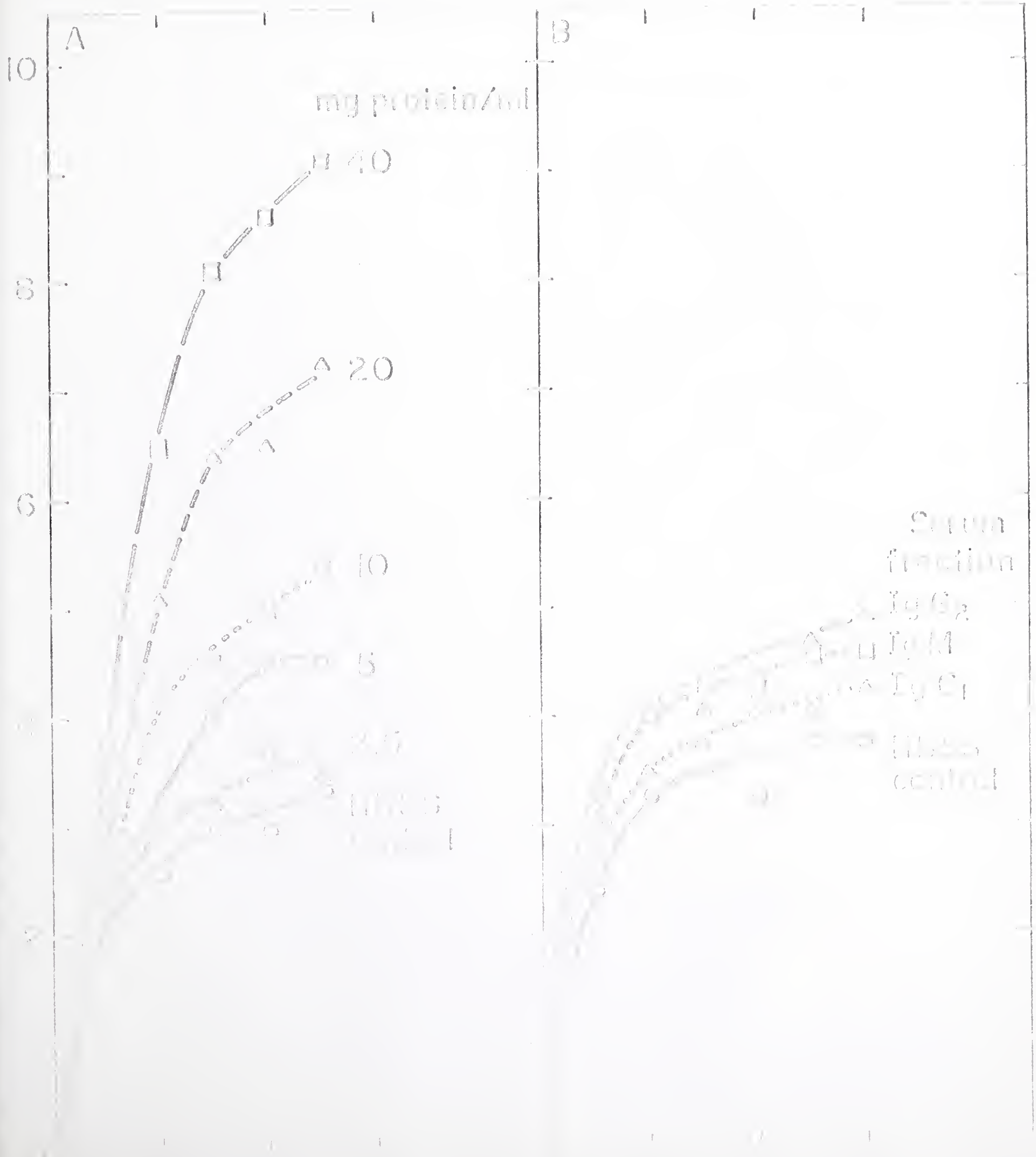

A
B
10
8
6
4
2
mg protein/ml
40
20
10
5
2.5
HBSS
control
Serum
fraction
IgG2
IgM
IgG1
HBSS
control

Fig. 3. Sparing effect of whole serum on trypanosome death rate in the presence of 200 μM pentamidine (Patton, C.L., unpublished data).

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