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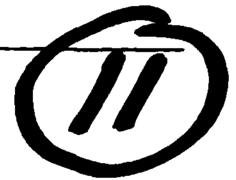

REPORT NUMBER 1

AD A095616

Trypanosoma b. rhodesiense (WRATat Serodeme): Purification and Characterization of Surface Antigens for the Vaccine Development Program

Annual Summary Report

Gerald R. Keilman

April 1980

Supported by
U.S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701

Contract Number: DAMD 17-79-C-9117

College of Veterinary Medicine
and Biomedical Sciences
Colorado State University
Fort Collins, Colorado, 80523

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REPORT DOCUMENTATION PAGE

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1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A095 616
3. RECIPIENT'S CATALOG NUMBER	
4. TITLE (and Subtitle) Trypanosoma b. rhodesiense (WRATat). Purification and characterization of surface antigens for Vaccine Development Program.	5. TYPE OF REPORT & PERIOD COVERED Annual Summary Report 1 August 1979 - 1 April 1980
	6. PERFORMING ORG. REPORT NUMBER Report Number 1
7. AUTHOR(s) Gerald R. Keilman, Research Associate	8. CONTRACT OR GRANT NUMBER(s) DAMD 17-79-C-9117
9. PERFORMING ORGANIZATION NAME AND ADDRESS College of Veterinary Medicine and Biological Sciences, Colorado State University, Fort Collins, Colorado 30523	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 62770A 3M162770A802 22 114.
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research and Development Command Fort Detrick, Frederick, MD 21701.	12. REPORT DATE April 1980
	13. NUMBER OF PAGES 33
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)	15. SECURITY CLASS. (of this report) Unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

African trypanosomes; T.b. rhodesiense WRATat; variant antigen; glycoprotein purification; glycoprotein characterization; lectins; affinity chromatography; immunosuppression;dexamethasone; prednisolone; progesterone; cyclophosphamide; total-body irradiation; anti-neutrophil serum; tunicamycin.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Variant-specific surface antigens (VSSA) from Trypanosoma b. rhodesiense WRATat clones have been purified using Con A-lectins affinity chromatography. The monecular weights of VSSA-2 and VSSA-12 have been estimated to be about 65,000 daltons by SDS-PAGE.

Proteolytic activity in the lysates was significantly inhibited by including PMSF or TLC in the lysing buffers. Treatment of the lysate with p-aminobenzamidine-6-aminohexanoic acid-sepharose was equally effective.

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Characterization of the antigens by classical techniques in now being pursued.

Low levels of parasitemia (3×10^8/ml) were overcome by disturbing the innate resistance of the rodent hosts with cyclophophamide, dexamethasone, prednisolone or anti-neutrophil serum prior to infection. These treatments resulted in infections peaking at greater than 10^9 organisms/ml.

Separation of the trypanosomes from blood cells was accomplished by treating the infected blood with an hemagglutinating lectin from *Maclura pomifera*. This innovation allowed us to decrease the volume of DEAE-cellulose to only twice that of the blood volume and to run the columns at 4 C.

Experiments with the antibiotic tunicamycin have demonstrated that trypanosomes can be inhibited by very low concentrations. Because this drug interferes with glycoprotein biosynthesis, it could be very useful in studying VSSA synthesis and degradation.

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ABSTRACT

The work performed under this contract relates to the development of techniques that accelerate the purification of the variant-specific surface antigens from clones of Trypanosoma b. rhodesiense WRATat. In addition, the purified antigens were to be characterized and supplied to the Immunology Section (WRAIR) for their Vaccine Development Program.

The low levels (3×10^8/ml) of parasitemia in normal and total-body irradiated rats hindered the efficient purification of the coat protein because too many animals had to be handled. Different immunosuppressive regimens were used aimed at perturbing the resistance of these hosts. Cyclophosphamide, dexamethasone, prednisolone, progesterone, and anti-neutrophil serum were tried. Only progesterone failed to enhance the level of the first wave of parasitemia, while the other treatments resulted in concentrations of bloodstream trypomastigotes greater than 10^9/ml. These observations allowed us to conclude that the innate immunity of the rats was critically important in achieving large numbers of trypanosomes at first peak. In addition, these organisms were of the inoculated type as determined by indirect immunofluorescence.

Separation of the trypanosomes from the blood cells was found not to be economical in time and money. The normal DEAE-cellulose procedure uses 10:1 ratio of DEAE-column volume to blood volume. By treating heparinized-blood with the Maclura pomifera lectin before applying the blood to the column, the ratio was reduced to 2:1. The column could also be run at 4 C which helps preserve the integrity of the surface antigens. Other lectins have been used with varying degrees of success. It can be projected that anti-rat red blood cell serum would work in place of the lectins, and that

cell elutriation techniques would be more desireable than ion-exchange chromatography for separating the trypanosomes from the agglutinates.

Proteolysis of the surface antigen was limited during the protein purification by including phenylmehtylsulfonylfluoride or tosyl-L-lysyl-chloromethane in the lysing buffer. Treatment of the lysate with the solid phase trypsin inhibitor, p-aminobenzamidine-6-aminohexanoic acid-sepharose was equally effective.

Concanavalin A-sepharose affinity chromatography was used to purify the antigens to near homogeneity in one day. The development of this technique was hampered by including Triton X-100 in the lysing buffer and in the Con A-sepharose column buffer. Removing this non-ionic detergent from the two buffer systems, led to the quantitative binding of the surface antigen to the affinity matrix. Using this technique, two antigens have been prepared and found to have unique molecular weights (68 K and 64 K daltons). More of the clones are being processed now to obtain significant comparative data.

The procedures have been developed to rapidly and efficiently purify the surface antigens from Trypanosoma b. rhodesiense WRATat clones. Characterization of the antigens by classical techniques is now being pursued.

FORWARD

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal, Resources, National Academy of Sciences-National Research Council.

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REPORT

I. Statement:

Chronic infection of humans and other mammals with trypanosomes is marked by recurring parasitemias. The organisms in each successive wave of infection have been shown to possess a surface "coat" that is antigenically unique from the ones associated with the earlier populations. This ability to overcome the host's immune response by selective expression of secreted glycoproteins represents the basis for the problem of antigenic variation. Because the extent of variation has yet to be determined, a potential for immunological prophylaxis exists through the use of polyvalent vacc ies and/or vaccines comprised of common, though minor, antigenic determinants. The obvious importance of these surface antigens to the survival of the trypanosome in the human host dictates that a thorough understanding of their biochemistry and biosynthesis be developed.

The program with which we are associated is directed toward the development of a useful vaccine against _Trypanosoma_ _b_. _rhodesiense_ (WRATat Serodeme). This hemoparasite was isolated and biologically cloned from the blood of a patient with symptoms of African sleeping sickness. Antigenically distinct clones were later obtained from this source and serve as primary stock for vaccine development. Our role is to supply the program with pure variant-specific surface antigens from the unique clones and the vital characteristics of each protein.

II. Experimantal Approaches:

In developing the production of quantitative amounts of these antigenic glycoproteins, two very important aspects had to be considered:

A. Large quantities of starting material of cloned trypanosomes must be obtained.

B. Protein purification techniques must be established to prevent sig-

nificant proteolysis from occurring.

The former problem was and is a real difficulty with the T.b. rhodesiense WRATat because they are a chronic strain, usually not appearing in the blood of the normal or total-body irradiated (TBI) rat host at a cell concentration greater than 3×10^8/ml. Several approaches to relieving this dilemma were considered:

1. Buy, infect, and process large numbers of rats.

2. Perfect techniques for quantitatively recovering the trypanosomes from infected blood.

3. Increase the parasitemia through stress regimens in addition to or separately from total-body irradiation.

The second difficulty of establishing purification techniques that are simple, rapid and that check proteolysis was proiritized in the following way:

1. These antigens are glycoproteins (1), and should bind to lectin-affinity matrices.

2. Proteolysis could be prevented by including specific or non-specific protease inhibitors in the lysis buffers and the processing buffers.

III. Results:

Currently, we are buying at 15 rats/clone weighing about 300 gm each to irradiate and infect. To help improve the recovery of the trypanosomes and maintain the integrity of their surface antigens. we have modified the DEAE-cellulose purification scheme of Lanham's (2). This change takes advantage of the fact that lectins do not agglutinate T.b. rhodesiense WRATat in whole blood unless the organisms are physiologically stressed, but the same lectins agglutinate rat red blood cells.

A lectin from the osage orange, Maclura pomifera (MP), was chosen because of its high agglutinating titer. Melibiose is its most active substrate, which has yet to be reported as a carbohydrate component of any

trypanosome coat protein. Initial studies with commercial MP-lectin had shown that 4 ng/ml of heparinized-rat blood was enough to agglutinate the rbc's. Therefore, we obtained several osage oranges, collected and extracted the seeds for the appopriate lectin (Fig. 1). Ten nanograms of MP-lectin were mixed per milliliter of heparinized, infected rat blood and allowed to incubate for 15 min/ 0 C. The blood was then applied directly to a DEAE-cellulose column equilibrated with PBSG (57 mM Na_2HPO_4, 3 mM NaH_2PO_4, 44 mM NaCl, 55 mM glucose, pH 8.0) at a ratio of blood volume to bed volume of 1:2 at 4 C (Fig. 2). The same buffer without glucose (PBS) was overlaid on top of the blood and then pumped through the column. The trypanosomes usually cleared the column in 30 minutes, although some organisms continue to wash through after that time. The recoveries ranged from 80 - 90% of the organisms applied. Many factors can effect the relative surface charge of the cells in an infected animal that will cause the trypanosomes to be retarded by an anion-exchange resin. Therefore, differences in recovery of parasites does occur.

The third approach we have taken to increase the efficiency of trypanosome recovery has been to try to interfere with the rat's primary immune response. Our aim was to suppress the immune system long enough to allow the tyrpanosomes to establish their niche without significant host interference. From preliminary experiments, infections in normal mice were seen to fluctuate unpredictably. When rats were subjected to lethal doses of Co^{60}-irradiation (800 rads), the parasitemias remained relatively stable but still of a titer lower than 3×10^8 trypanosomes/ml. Levels of the circulating white blood cells were taken to determine that the irradiation regimen was effective (Table 1). By the third day post-irradiation, the level of wbc's in the peripheral circulation dropped to less than 10% of

normal values. The neutrophils (segments) decreased to about 30% of normal and the lymphocytes to 2.5%. These observations led us to conclude that the neutrophils were the controlling cell type of the host that were responsible for the plateau effect.

Experiments were designed to interfere with the innate response of the rodent hosts (Fig. 3) and compared to TBI and cyclophosphamide (CYCLO) immunosuppressed animals during the time it was required to determine the first peak of parasitemia. The data showed that treatments with the two glucocorticoid compounds dexamethasone (DEX) and prednisolone (PRED) and anti-neutrophil serum (ANTI-PMN) resulted in parasitemias peaking at about 2×10^9/ml. Because steroid compounds have multiple effects on cellular physiology, direct correlations are hard to establish. However, it is well documanted that neutrophil adherence and migration are decreased shortly after steroid treatment (3,4,5 and 6).

The sex hormone progesterone (PROG) was used to determine if steroids in general could increase the levels of parasitemia. The first group of animals received 2 mg/100 gm body weight and the second group was injected at 8 mg/100 gm body weight with progesterone. The latter regimen was used to control the one-fourth shorter half-life of progesterone compared to dexamethasone. Progesterone (Tables 2 and 3) treatment resulted in an equivalent increase in the number of circulating neutrophils to that of the other steroids. However, the levels of parasitemia in the treated animals never exceeded 3×10^8/ml. From these data, we concluded that the dexamethasone effect of increasing the plateau level of the parasitemia of _T. b. rhodesiense_ WRATat was not solely related to its effect on mobolizing neutrophils in the circulation.

Treatment of normal rats with rabbit anti-rat-neutrophil serum gave the best results of all the regimens. Animals were injected with 0.5 ml

each of anti-PMN serum on three successive days. On Day 2, each animal was infected with stabilated trypanosomes four hours after the second injection. The schedule allowed the parasite level in the blood to reach 2×10^9/ml. The rats still showed an induced humoral response as the parasites were cleared from the circulation as in a normal, untreated animal.

The technique of purifying the surface antigens by affinity chromatography was suggested by the observation that these are indeed glycoproteins (1). We combined this method with DEAE-cellulose fractionation of the cell lysates, as these proteins do not bind to the anion-exchange resin at pH 8.0.

Until recently, we had been lysing the trypanosomes by thawing the frozen pellets in the presence of 1.5% Triton X-100. The clarified lysates were then applied to the two columns after a 1:9 dilution with PBS. The coat protein did not stick to the DEAE-cellulose nor to the Con A-sepharose column. After several months of purifying, synthesizing, and purchasing different Con A and lentil lectin matrices, we discovered that these glycoproteins will not bind to the lectin columns in the presence of Triton X-100. Figure 4 shows our most recent purification of the main glycoprotein from T.b. rhodesiense WRATat-2. This protein was obtained from sheared lysates of cells in the presence of phenylmethylsulfonylfluoride (PMSF), a serine-active site protease inhibitor, and in the absence of Triton X-100. Almost all of the surface antigen bound and eluted from the Con A-sepharose column under these conditions. Triton X-100 or its equivalent (Nonidet P-40; 7) has been used in affinity chromatography techniques to minimize the non-specific, hydrophobic binding. Because the glycoproteins from the WRATat clones do not bind to the lectin columns with Triton X-100 in the buffers, we have concluded that the carbohydrate moiety is closely associated with the hydrophobic domains of these proteins. Both the carbohydrates and the hydrophobic regions are buried in the micells of the detergent and are not

available for interaction with the lectins. Currently, we are purifying the other surface antigens from lysates lacking non-ionic detergents accordingly:

1. Thaw washed pellets in the presence of a protease inhibitor in phosphate buffered saline (PBS) pH 8.0/4°C.

2. Shear lysate with syringe passage. Use syringe needles 18G through 27G/4°C.

3. Centrifuge lysate at 30,000 x g/30 min/4°C (F_1).

4. Apply the supernatant fluid directly to the DEAE-cellulose column equilibrated with PBS, pH 8.0/4°C.

5. Collect (F_2) and dialyze (F_4) the flow-through fractions against 20mM Tris HCl, pH 7.4, 500 mM NaCl, 0.1 M $MnCl_2$ and 0.1 mM $CaCl_2$, the Con A-sepharose column buffer. Elute DEAE with IM NaCl (F_3).

6. Apply sample to the lectin column and wash the sample through with the Tris buffer. Flow-through fractions= F_5.

7. Elute the bound molecules with 100 mM α-methyl-mannopyranoside in Tris-buffer, F_6 (see Table 4).

The total recovered glycoprotein represents about 4% of the initial soluble protein.

Figure 5 represents the results of an immunodiffusion experiment demonstrating that our procedure purifies authentic WRATat-2 antigen, that some antigen passed through the Con A-sepharose column; that the proteins eluted from the DEAE-cellulose column with IM NaCl lack that antigen.

The molecular weights of WRAT-2 antigen and WRATat-12 antigen (previously purified) were estimated by SDS-PAGE (8) to be 64,000 and 68,000 respectively. Gel permeation chromatography using HPLC and a TSK 3,000-SW column was performed on WRATat-12 glycoprotein. Molecular weight analyses of the native protein were inconclusive, but the protein eluted just after BSA, suggesting that it

was slightly smaller than BSA.

During the processing of the WRATat-12 antigen on this column, a very important observation was made. The purified protein underwent association-dissociation in concentrated solutions (Figure 6). The Con A-purified protein solution was concentrated in 10 mM KP buffer with 100 mM KCl and 0.02% NaN_3. This sample was injected into the TSK 3000-SW column and resolved into three peaks at 900 psi at 1 ml/min/25°C. The second (dimer) peak was concentrated and reapplied to the same column 4h later. Again, three peaks were resolved. Association phenomena are not uncommon for purified proteins in concentrated solutions. However, our observation does suggest that the mechanism for coating the trypanosomes *in vivo* could be related, as these proteins are also found at high concentrations on the surface of the organism.

The resolving power and speed of HPLC molecular sieving became apparent in these studies. Attempts were made to use this technique to purify the surface antigen of the trypanosomes. Because the coat glyco-proteins associate into multimers in concentrated solutions, we found this an unrewarding procedure. Once the nature of the interaction is understood, it may become possible to prevent or stimulate the process so that HPLC can be used more effectively.

As indicated above, proteolytic activity in the trypanosome extracts was minimized by thawing the frozen pellets in PBS containing freshly dissolved phenylmethylsulfonylfluoride (PMSF, 1mM). Trypsin-like activity appeared to be the only significant problem. Tosyl-L-lysyl-chloromethane (TLC) was also used in lysing buffers with equal success. The latter was the preferred inhibitor, but its cost was significantly greater. We have recently synthesized p-aminobenzamidine-6-aminohexanoic acid-sepharose, a solid-phase trypsin inhibitor and used it just after the lysing step. This matrix worked as well as the previous methods and has the advantage of physically removing the proteases from the lysates.

We have initiated some studies on the antibiotic tunicamycin (TM). The reagent is known to inhibit glycoprotein synthesis and will be used by this laboratory in an attempt to study various aspects of VSSA biosynthesis, translocation and degradation.

Two types of experiments were performed using TM:

1. Purified trypanosomes were incubated with TM for 120 min in RPMI + 10% FCS at either $0^{o}C$ or $37^{o}C$. Our first experiment showed that all TM-treated parasites were "non-infectious". A second similar experiment gave variable results, which we discovered was caused by the instability of TM in our storage solutions. The recent development of an HPLC purification procedure now allowed us to characterize the stable drug (Fig. 7) and select the fractions we desired. Drug obtained in this manner was used to repeat the initial experiment at $0^{o}C$ (only). Again, no apparent infections from TM-treated trypanosomes occurred, even though the parasites were still viable after 24 hours in the presence of TM/$4^{o}C$.

2. Mice that had been infected with parasites incubated at $0^{o}C$ or $37^{o}C$ for 120 min in RPMI + 10% FCS were later injected with 5 μg each of TM (Fig. 8) when the infection had reached its peak. In all cases TM seemed to potentiate clearance of the trypanosomes and prolonged the period until recurrence was noted. Although the clinical use of this potent drug is doubtful, it is apparent that the trypanosomes are susceptible to its activity.

IV. Conclusion:

The work completed in the first nine months has been successful in every aspect except a temporal one. The latter refers to the slow resolution of the proper conditions for binding the surface antigens to the affinity matrix, Con A-sepharose. This problem had slowed up all the proposed analyses on the glycoproteins because there were no other techniques that were as effective.

Although preparative isoelectric focusing has been used by others, it requires expensive equipment, reagents and long equilibration time. The lectin-affinity column facilitated the purification (99%) of the WRATat-2 surface antigen in one day. The expense in time resolving that problem will be alleviated by the rapid processing of the remaining antigens and the awareness that the carbohydrate portion of them is closely associated with the hydrophobic domain.

Significant advances have been made in our laboratory:

1. Glucocorticoid drugs elevate the initial wave of parasitemia three- to five-fold over normal or TBI-animals. These high concentrations will allow us to harvest more trypanosomes from fewer rats.

2. We improved the purification of trypanosomes from whole blood by pretreating the blood with a potent hemagglutinating lectin from Maclura pomifera. This innovation has allowed us to separate the trypanosomes on one-fifth the amount of DE 52. The fractionation can take place at 4°C which effectively decreases proteolytic activity.

3. Adaptation of a solid-phase protease inhibitor has allowed us to purify these glycoproteins in the absence of serine-reactive PMSF or expensive TLC.

4. The work initiated with tunicamycin offers us the important information that the carbohydrates are asparagine-linked and that the inhibition of trypanosome glycoprotein biosynthesis in the host can result in the removal of the parasite from the circulation.

5. We have learned that the carbohydrate portion of these antigens is probably associated with the hydrophobic domain. Chromatography on hydrophobic matrices is suggested and offers an approach to the purification of that domain as well as the intact glycoprotein.

6. High performance liquid chromatography through permeation beds has

shown us that the pure glycoproteins associate and dissociate freely in solution. This observation suggests the mechanism of "coating" in these parasites and is stimulus for more detailed studies.

V. Recommendations:

We feel that the next few months of work will result in significant information on the chemistry of the surface glycoproteins of T.b. rhodesiense WRATat. Although the extent of analysis will be less than originally proposed, the qualitative nature will not change. The areas of increasing parasitemias, parasite-blood cell separation, tunicamycin inhibition of glycoprotein synthesis, and parasite development in tunicamycin treated animals all need further investigation. Currently, we are concentrating our efforts on determining the chemical nature of the main antigenic glycoproteins of T.b. rhodesiense WRATat.

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Table 1.

EFFECT OF CO^{60}-IRRADIATION ON WBC'S OF RATS (800 RADS) N-11

	Day 0	Day 1	Day 3
Total WBC's	17,341 (100%)	4,428 (100%)	1,296 (100%)
#Segments	3,330 (19%)	3,423 (77%)	946 (73%)
#Lymphocytes	13,145 (76%)	1,001 (23%)	333 (26)
#Monocytes	676 (4%)	-	10 (1%)
#Eosinophils	173 (1%)	4 (0.1%)	-
#Bands	17 (0.1%)	-	7 (0.5%)

Table 2.

EFFECT OF PROGESTERONE ON WBC'S OF RATS (2mg/100g I.M.) N=6

	Day 0	1 Hour	2 Hour
Total WBC's	19,600 (100%)	24,580 (100%)	21,261 (100%)
#Segment	6,272 (32[illegible])	5,924 (24.1%)	8,887 (41.8%)
#Lymphocytes	12,054 (61.5%)	16,886 (68.7%)	11,435 (53.8%)
#Monocytes	529 (2.7%)	688 (2.8%)	383 (1.8%)
#Eosinophils	549 (2.8%)	737 (3.0%)	319 (1.5%)
#Bands	131 (.67%)	320 (1.3%)	176 (.83%)

	3 Hours	4 Hours	5 Hours
Total WBC's	19,439 (100%)	19,637 (100%)	20,286 (100%)
#Segments	7,853 (40.4%)	9,779 (49.8%)	12,415 (61.2%)
#Lymphocytes	10,653 (54.8%)	8,679 (44.2%)	7,222 (35.6%)
#Monocytes	349 (1.8%)	530 (2.7%)	203 (1.0%)
#Eosinophils	349 (1.8%)	432 (2.2%)	284 (1.4%)
#Bands	233 (1.2%)	236 (1.2%)	136 (.67%)

	6 Hours	24 Hours (1 Day)
Total WBC's	15,707 (100%)	24,268 (100%)
#Segments	8,717 (55.5%)	7,037 (29.0%)
#Lymphocytes	5,969 (38.0%)	16,065 (66.2%)
#Monocytes	196 (1.25%)	405 (1.67%)
#Eosinophils	196 (1.25%)	558 (2.3%)
#Bands	628 (4.0%)	201 (.83%)

Table 3.
EFFECTS OF PROGESTERONE ON WBC'S OF RATS (8mg/100g I.M.) N=6

	Day 0	1 Hour	2 Hours
Total WBC's	18,065 (100%)	20,705	16,135
#Segments	3,071 (17%)	7,039 (34%)	7,261 (45%)
#Lymphocytes	13,910 (77%)	13,042 (63%)	8,390 (52%)
#Monocytes	181 (1%)	207 (1%)	97 (.6%)
#Eosinophils	361 (2%)	828 (4%)	403 (2.5%)
#Bands	181 (1%)	207 (1%)	161 (1%)

	3 Hours	4 Hours	5 Hours
Total WBC's	17,118	17,624	17,521
#Segments	10,613 (62%)	11,456 (65%)	11,914 (68%)
#Lymphocytes	6,162 (36%)	5,640 (32%)	5,256 (30%)
#Monocytes	29 (.17%)	176 (1%)	87 (.5%)
#Eosinophils	274 (1.6%)	88 (.5%)	87 (.5%)
#Bands	171 (1%)	176 (1%)	87 (.5%)

	6 Hours	24 Hours
Total WBC's	18,200	24,528
#Segments	13,468 (74%)	3,679 (15%)
#Lymphocytes	6,734 (37%)	20,358 (8.3%)
#Monocytes	328 (1.8%)	368 (1.5%)
#Eosinophils	0 (0%)	392 (1.6%)
#Bands	364 (2%)	0 (0%)

Table 4: Summary of Purification of Surface Antigen from T.b. rhodesiense (WRAT-2)

Fraction	Total Volume (ml)	Protein* (mg/ml)	Total Protein (mg)	Yield
F_1; 30,000xg supernatant fluid	1.35	6.9	9.3	100%
F_2; DEAE-cellulose flow-through	6.8	0.9	6.1	66
F_3; 1M NaCl DEAE-eluate	6.0	0.4	2.4	26
F_4; Dialyzed F_2	6.2	0.7	4.3	46
F_5; Con A-sepharose flow-through	9.0	0.35	3.1	33
F_6; 100 mM methylmannopyranoside Con A-sepharose-eluate, peak-fraction	1.0	0.30	0.3	3.2

*Lowry protein assay

LEGENDS TO FIGURES

Figure 1: Purification of the lectin from Maclura pomifera (Osage orange). Seeds were rendered from the fruit and completely processed in four days.

Figure 2: Diagram of a glass column used in the DEAE-cellulose separation of trypanosomes from rat blood cells. The inclusion of the glass wool layer and the filter cloth (Miracloth, Chiopee Mills, Inc. - New Jersey) allowed higher flow rates on blood samples that have been agglutinated with lectins.

Figure 3: Growth of Trypanosoma b. rhodesiense (WRATat Serodeme) in female rats that had undergone stress regimens prior to infection: Upper left panel, untreated animals; upper middle panel (TBI), rats exposed to 900 rads of gamma irradiation 3 days prior to infection; upper right panel, (CYCLO), animals treated 6 h before infection - 15 mg/100 gm body weight of cyclophosphamide; lower left panel (DEX), pre-treatment (6 h) with dexamethasone at 2 mg/100 gm body weight; lower middle panel (PRED), pre-treatment with prednisolone at 2 mg/100 gm body weight; lower right panel (ANTI-PMN), animals treated for 2 days prior to infection with rabbit anti-polymorphonuclear leukocyte serum.

Figure 4: Purified $VSSA_2$: Lane 2 shows the purity of the Con A-sepharose eluate from lysates of T.b. rhodesiense WRATat-2. The middle upper band in this lane is the dimer of the surface antigen, which dissociates when the samples are heated in the presence of SDS. Lanes 1 and 8 contain the standard proteins: phosphorylase b, transferrin, bovine serum albumin, carbonic anhydrase, and cytochrome c (at dye front); lane 3, Con A-sepharose column flow-through; lane 4, sample applied to the Con A column; lane 5, 1 M NaCl eluate of the DEAE-cellulose column; lane 6, DEAE flow-through fractions; lane 7, sample applied to the DEAE column. SDS-PAGE (10%) according to Laemmli (8).

Figure 5: Ouchterlony double diffusion: Center well contained rabbit anti-WRATat-2 surface antigen (provided by K. Esser, WRAIR); A, Con A-sepharose eluate (see lane 2, Fig. 4); B, Con A-sepharose flow-through (see lane 3, Fig. 4): C, bovine serum albumin; D, 1 M NaCl eluate of DEAE-cellulose column (see lane 5, Fig. 4); E, sample D after passing through a Con A-sepharose column; F, Con A-sepharose eluate of sample D.

Figure 6: High performance liquid chromatography (HPLC): A sample of Con A-sepharose-purified glycoprotein from T. b. rhodesiense WRATat-12 was concentrated in an ultrafiltration device with a PM-10 filter (Amicon Corp. - Massachusetts). The solution was applied to a TSK 3,000-sw permeation bed and resolved at 900 psi with 10 mM KP buffer (100 mM KCl). The fractions in the dimer position were collected, concentrated, and re-applied to the same column. Processing time was 4 h/ 4 C. SDS-PAGE of the monomer and dimer pooled fractions showed only one protein band in position with the VSSA-12 glycoprotein.

Figure 7: HPLC: Resolution of tunicamycin samples in methanol-water (3:1) on an Ultrasil-ODS C_{18} reverse-phase column (Altex Scientific, Inc. - California). Sample A = lot # 361-26E-235A. Sample B = lot # 361-26E-250A.

Figure 8: Tunicamycin (TM) treatment: C57Bl/6J mice were infected with T. b. rhodesiense WRATat-2 that had been incubated at either 0 C or 37 C/ 120 min in RPMI + 10% FCS. All mice except the controls were injected with a total of 5 ug of TM on the third day after infection. Upper panels, mice infected with trypanosomes pre-incubated at 0 C; lower panels, trypanosomes incubated at 37 C.

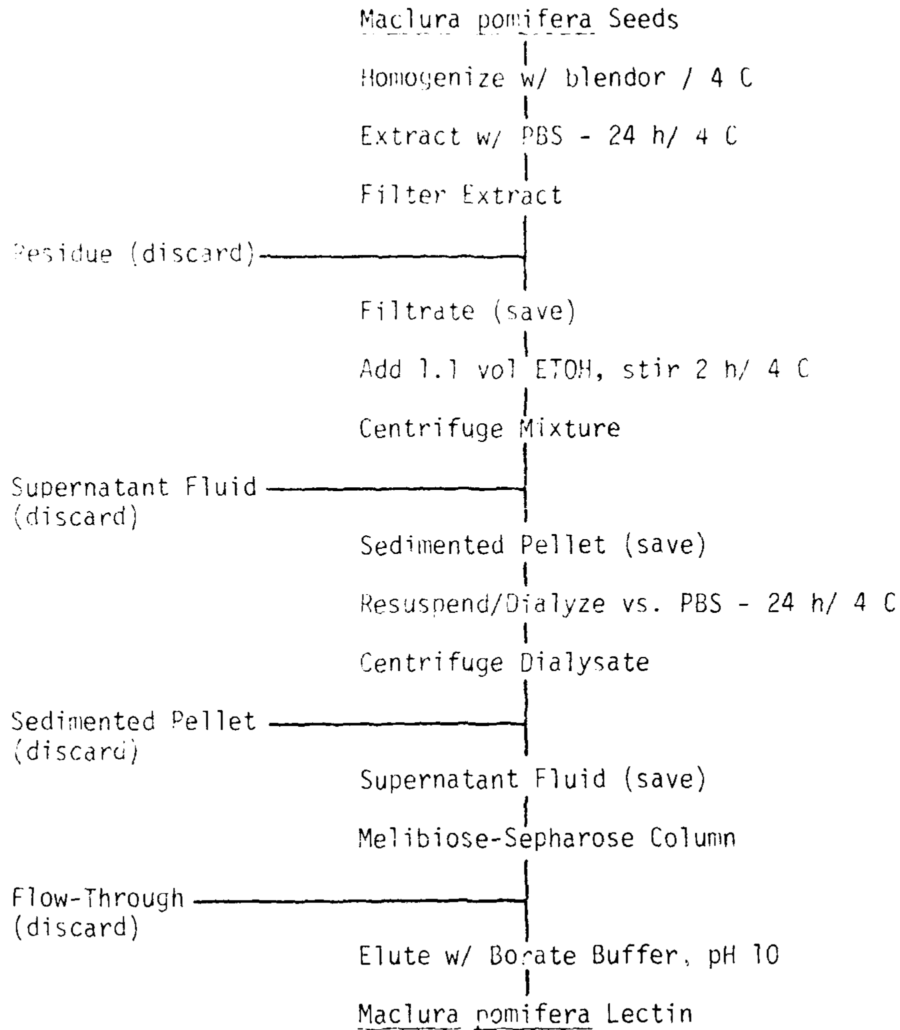


FIG. 1

DEAE COLUMN

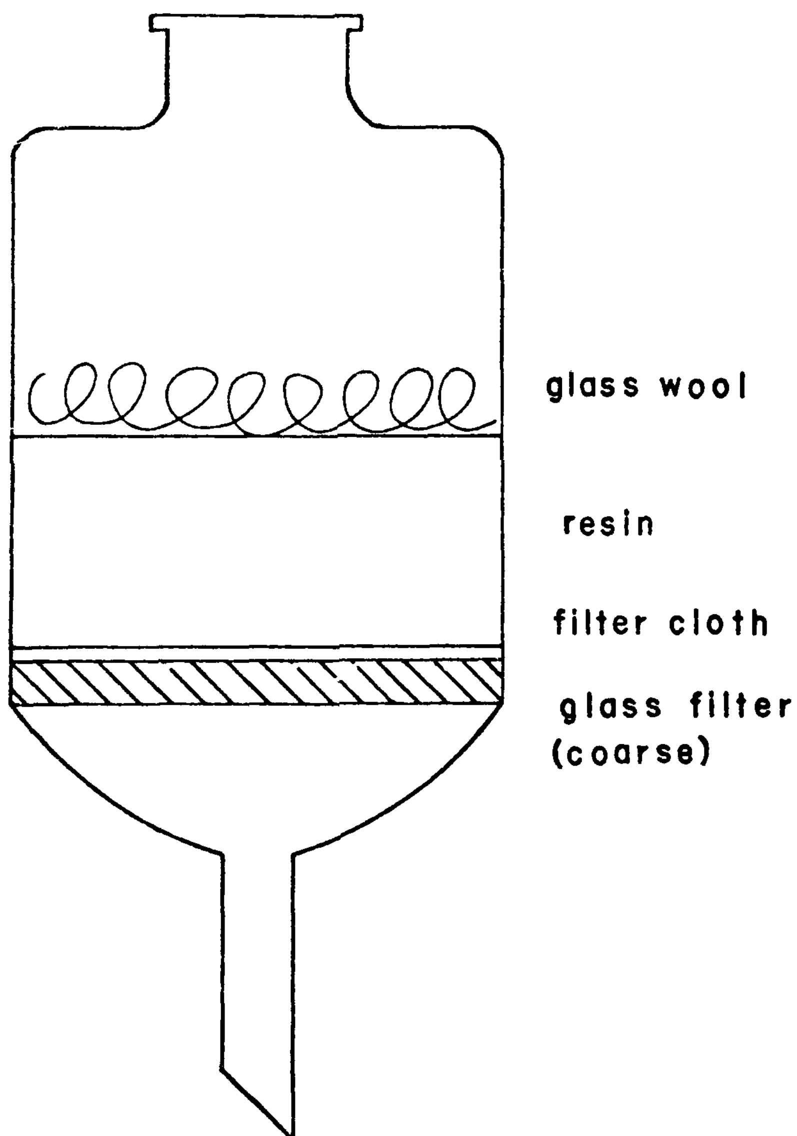


FIG. 2

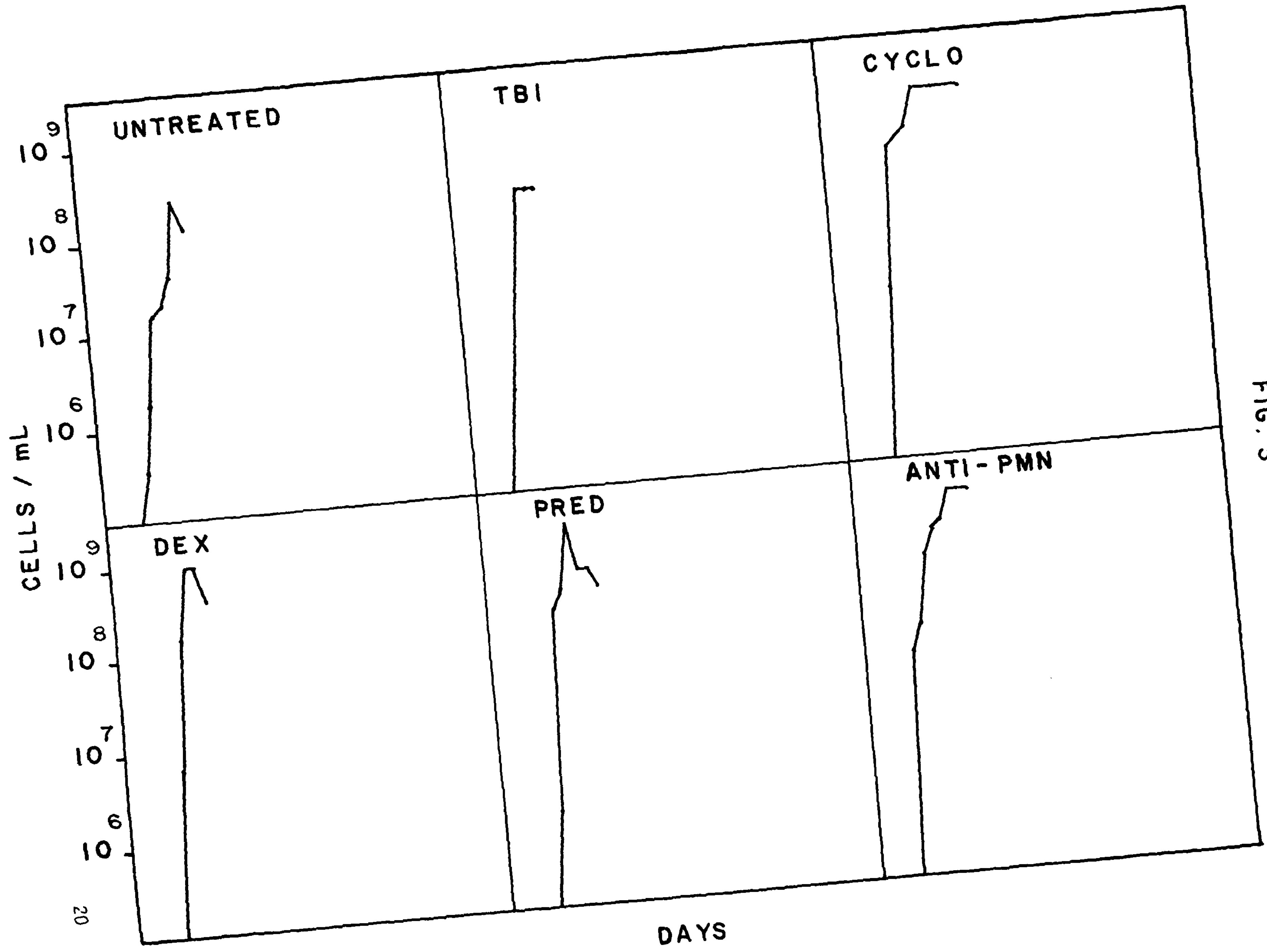


FIG. 3

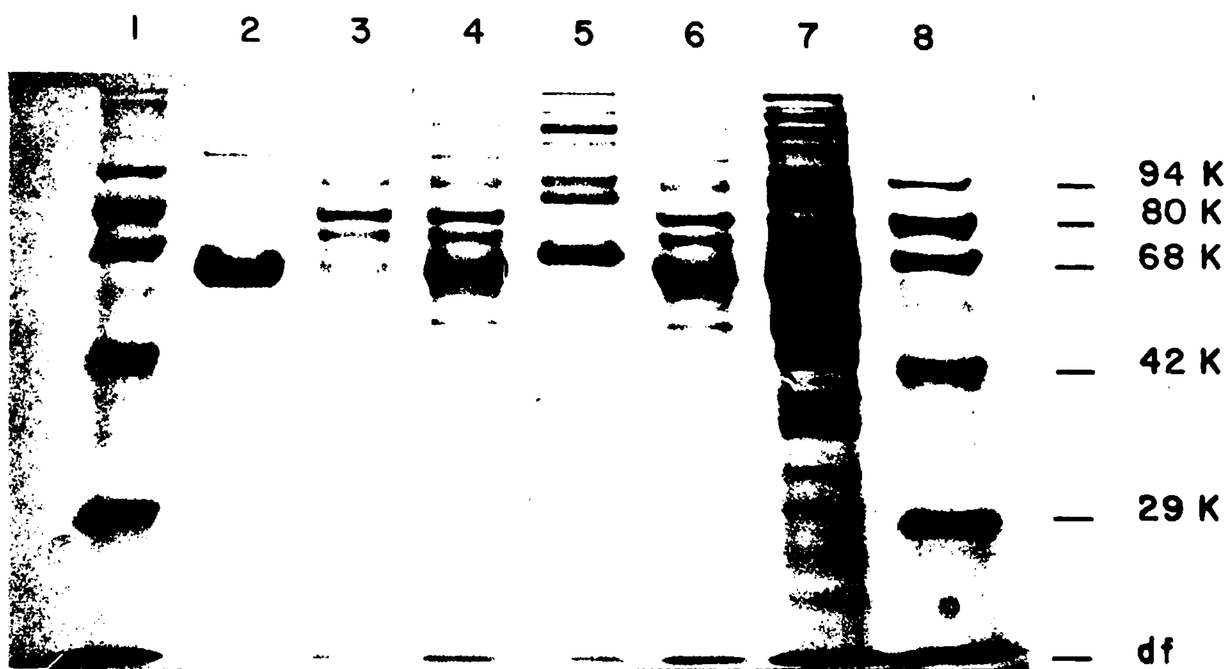


FIG. 4

IMMUNODIFFUSION

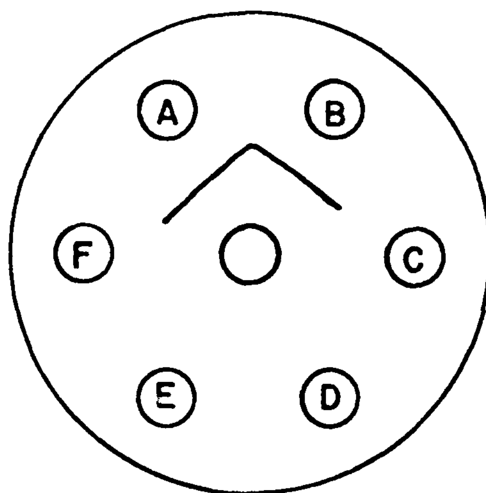


FIG 5

HPLC OF WRATat-12 ANTIGEN

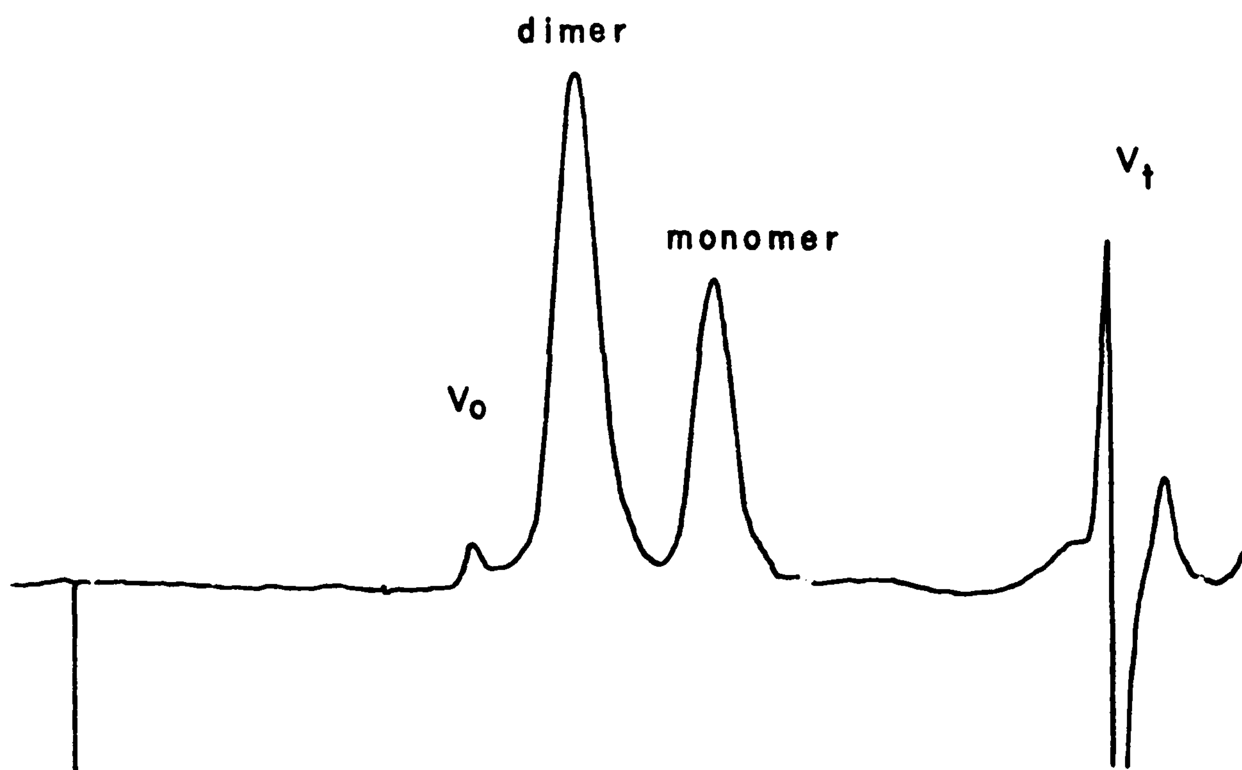
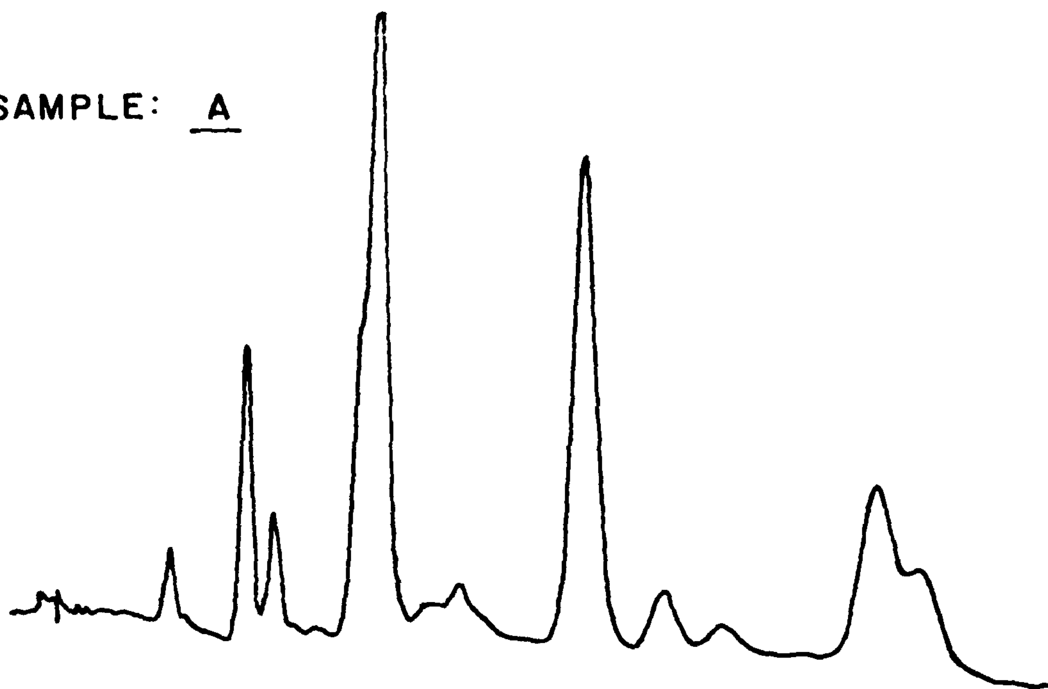


FIG. 6

HPLC OF TUNICAMYCIN



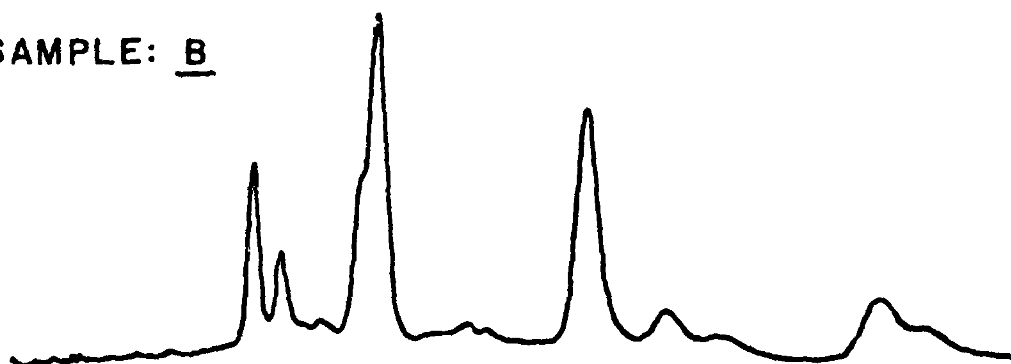
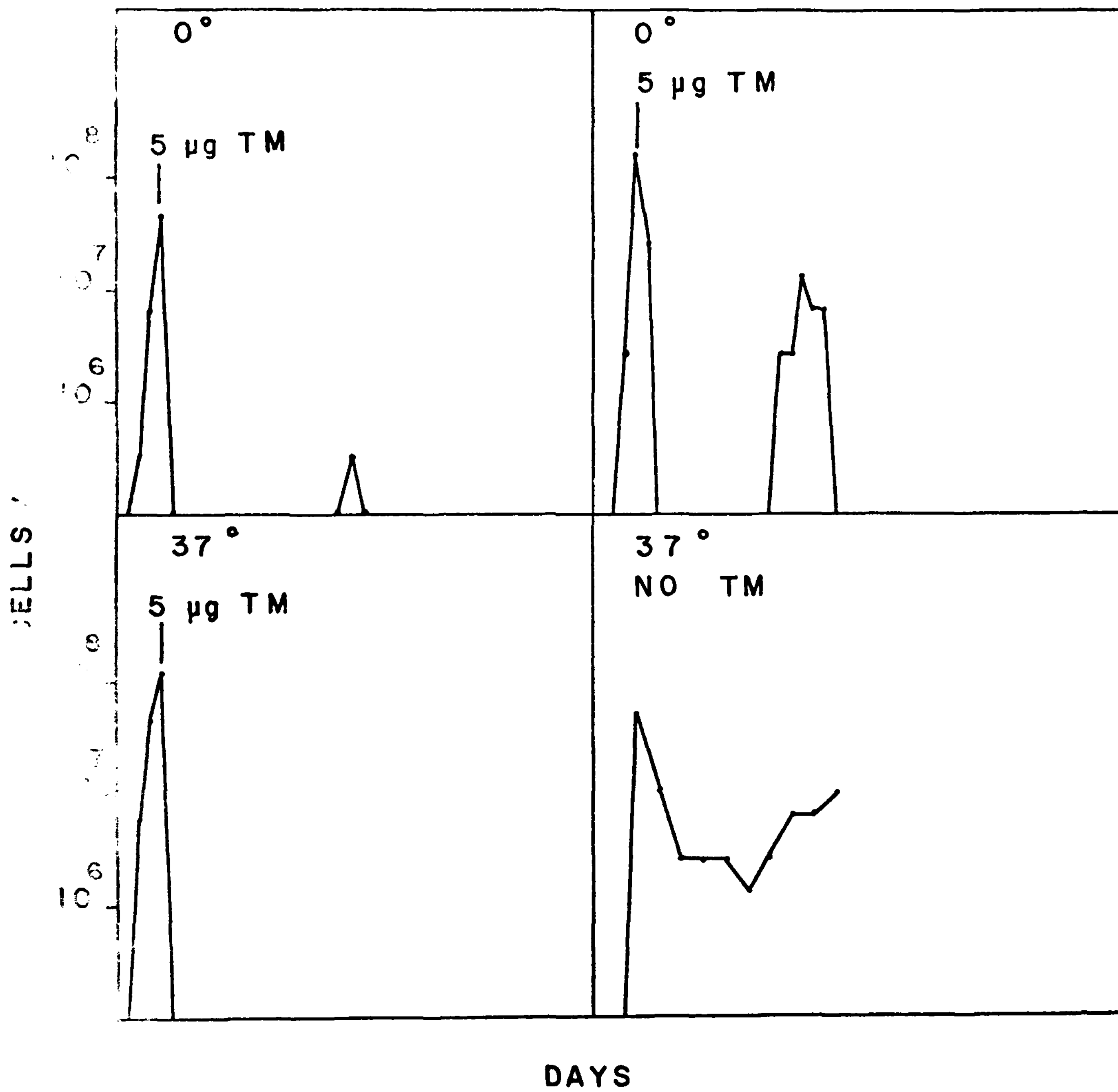


FIG. 7

FIG. 8



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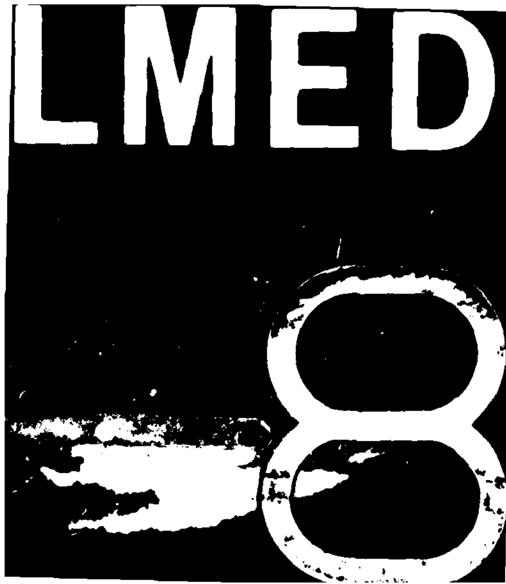
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