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REPORT NUMBER ONE

DRUG DESIGN RELATING AMEBICIDES TO INHIBITION OF PROTEIN SYNTHESIS (U)

FINAL REPORT

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New York University School of Medicine

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We asked the question, "Is active protein synthesis required in order for emetine to exert its amebicidal effect?" The answer appears to be "no." First a study of the effect of emetine on protein synthesis in E. histolytica was made on log phase amebas as compared to stationary phase amebas. ~~It was found that~~ sensitivity to emetine ~~is~~ was maintained independently of the rate of protein synthesis. Furthermore, both stages of amebas had the same capacity to bind emetine labeled with tritium to ribosomes. The binding of H^3-emetine

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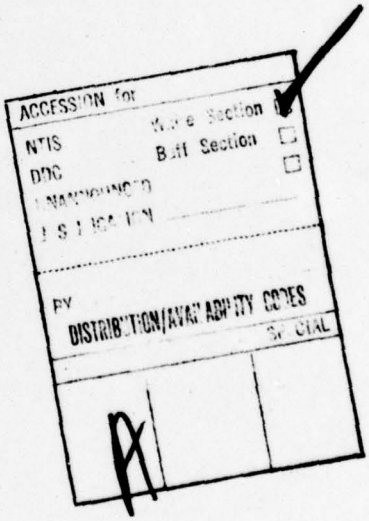
20. Abstract (cont.)

was not affected by the presence of certain drugs that interfere with energy metabolism, protein synthesis and/or ribosomal function, e. g., dinitrophenol, puromycin, shloroquine and acriflavin. In "chase" experiments it was shown that the stability of the emetine-ribosome binding is due in part to a hydrogen bonding reaction of the C_1 atom of the emetine molecule with the chain elongation site. Finally, evidence was obtained that the capacity to bind emetine provides a basis for conferring drug resistance in amebas. A direct correspondence was found between the degree of drug resistance and the number of binding sites for emetine.



FOREWARD

The author intends to submit for publication in a suitable journal most of the information in this report.

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SUMMARY

Having demonstrated in a previous paper (1) that emetine is amebicidal by virtue of inhibition of protein synthesis, we conducted some experiments to gain some further insight into the mechanism of action of emetine on E. histolytica. We asked the question, "Is active protein synthesis required in order for emetine to exert its amebicidal effect?" The answer appears to be "no".

This was demonstrated in several ways on cultured forms of amebas. First a study of the effect of emetine on protein synthesis was made on log phase amebas as compared to stationary phase amebas. In the latter the rate of protein synthesis is significantly lower. It was found that sensitivity to emetine, i.e. degree of inhibition of protein synthesis, is maintained independently of the rate of protein synthesis. Furthermore, both stages of amebas had the same capacity to bind emetine (labeled with tritium) to ribosomes. The binding of H^3-emetine was not affected by the presence of certain drugs that interfere with energy metabolism, protein synthesis and/or ribosomal function, e.g. dinitrophenol, puromycin, chloroquine and acriflavine. High concentrations of EDTA combined with puromycin which supposedly disaggregate ribosomes into their subunits caused a 50% reduction of binding.

Some other aspects of emetine binding were revealed. In "chase" experiments the ribosomes of intact amebas were first allowed to bind with H^3-emetine or H^3-isoemetine and then exposed to relatively high concentrations of unlabeled emetine. Labeled isoemetine was displaced almost completely, while no displacement of H^3-emetine occurred. This shows that the high stability of the emetine-ribosome binding is due in part to a hydrogen bonding reaction of the C_1 atom of the emetine molecule with the chain elongation site.

1. Entner, N. and Grollman, A.P. "Inhibition of Protein Synthesis:

Finally, evidence was obtained that the capacity to bind emetine provides a basis for conferring drug resistance. The LA strain (original Laredo strain) is about 10 times more resistant to emetine than the regular F-22 strain and it binds at least twice as much emetine per ameba. When the LA strain is grown and tested at room temperature, the resistance to emetine increases at least another 20-fold, and interestingly there appears a corresponding increase, at least 8-fold, in the number of binding sites for emetine, most of which are probably not involved in protein synthesis.

The work reported here involves some preliminary unpublished experiments concerning the nature of action of the drug emetine on Entameba histolytica. In a previous publication (1) we demonstrated that emetine and some structurally related drugs exert their amebicidal effect by specifically inhibiting protein synthesis. We also investigated some of the structural requirements in the emetine molecule necessary for biological activity. During part of the time, supported by the U. S. Army Medical Research and Development Command, we engaged in a literature survey which enabled us to propose the synthesis of new amebicides structurally related to emetine. These proposals were submitted to the above group for consideration of continued support but could not be funded.

In the work reported here we asked the question - "Does the specific action of emetine require an active protein synthesizing system to be going on - or any other active biochemical reactions?" The answer appears to be "no".

The following experiments were carried out: (1) A comparison of emetine action on intact amebas harvested during logarythmic phase of growth to those harvested at stationary phase. The activities investigated were rate of protein synthesis and binding of tritium-labeled H^3-emetine to amebal ribosomes. (2) Some other drugs involved in macromolecular synthesis were tested to see if they had an effect on emetine binding. (3) Some evidence for a basis for drug resistance in E. histolytica was found. (4) An experiment on the stability of the drug-enzyme (on ribosome) complex was carried out.

RESULTS

Protein Synthesis and Emetine Binding

Amebas harvested during the logarythmic (log) phase of growth show the maximal rate of protein synthesis. Stationary phase amebas obtained accord-

ing to previously established growth conditions) show a considerable variation in the rate of protein synthesis. It is always slower than the rate in log phase amebas, and on a rare occasion one can obtain a population that carries out no protein synthesis at all during the 3 hour period in which the experiment is carried out (See Table 1-B). It should be mentioned that in each experiment for comparison, the "log phase" and "stationary phase" were obtained from the same initial inoculum. Only the harvesting time was different. In Table 1 it can be observed that the sensitivity to emetine is unaffected by a change in the rate of protein synthesis. (Differences in drug sensitivity between the two strains, F--22, a regular strain and LA, the Laredo strain are not as clear cut as has previously been observed.)

If drug sensitivity is unaffected, one might expect that binding of emetine to ribosomes would be the same regardless of the rate of protein synthesis and the stage of growth. Binding of H^3-labeled emetine to amebal ribosomes indeed appears to be independent of protein synthesis (See Table 2 - controls) (also puromycin experiments). There is no reduction of binding capacity in stationary phase amebas. At the same time some experiments were carried out to determine whether H^3-emetine binding could be affected by the presence of some drugs that interfere with other aspects of macromolecular synthesis.

None of the drugs tested significantly altered the binding of emetine, although the presence of chloroquine usually caused some decrease in the amount bound. Chloroquine and acriflavin at 10^{-4} gms/ml were added at concentrati that had been found to be amebicidal. The concentration of dinitrophenol was sufficient to uncouple high energy bonds, although it is possible that the compound was not permeable to the amebas.

The effect of puromycin was tested separately (Table 3). This compound by taking the place of an activated amino acid causes termination of any nascent polypeptide chains, which then is stripped off of the ribosome. H^3-emetine binding was unaffected even when amebas were preincubated with puromycin for 1 hr. (Table 3). When EDTA is added with puromycin, a condition for disaggregating ribosomes into their subunits the binding of H^3-emetine was decreased approximately 50% (Table 4). These conditions are somewhat harsh, and the decrease of binding could be an indirect effect resulting from inhibition of some other functions. The results would indicate that separate 60 s subunits still maintain a capacity to bind emetine.

Nature of Bond

Emetine and cycloheximide have certain analogous structural confirmations and exert their specific inhibitory effect by binding at the chain elongation site on the 60 s subunit of the ribosome. They differ however in that the bond of emetine with ribosomes is irreversible, while that of cycloheximide is reversible. The latter can be washed out.

When emetine-ribosome complexes are brought to an alkaline pH, the free base can be extracted into organic solvents such as toluene. These suggests that a covalent bond between emetine and ribosome does not occur.

We were thus led to test whether or not bound emetine will exchange with externally added emetine - in a so-called "chase" experiment. A similar experiment was conducted between bound isoemetine and externally added emetine. The results are shown in Table 5. Bound emetine cannot be "chased" while bound isoemetine is almost totally displaced. This allows one to relate binding to the active site of enzyme. Two of the major binding sites in the emetine molecule are the two N moieties. The "C_1" carbon atom of emetine is that which engages the active site of the ribosomal enzyme that carries out chain elonga-

tion by adding activated amino acid to the growing peptide chain. Emetine differs from isoemetine only in the bond angle that the C_1 atom makes with its single H atom. While the binding capacity of the two epimers appears to be the same, isoemetine does not inhibit protein synthesis and is not amebicidal (1). From these results it would appear that the stability (irreversibility) of emetine binding to ribosome is due to hydrogen bonding between C_1 and the active enzyme site which then causes inhibition of enzyme activity.

Emetine-binding and Drug Resistance

The LA-type strains are well-known to be more resistant to the action of emetine than the regular strains of E. histolytica. They require approximately 10X more drug for 100% killing.

In Fig. 1 it is shown that resistance is not due to differences in permeability. At a given concentration of drug a plateau for bound H^3-emetine is reached rapidly 10 and 30 minutes in F-22 and LA strains, respectively. However, at the concentration used not all potential binding sites are occupied. The total number of potential binding sites are revealed when the amebas are exposed to varying concentrations of drug. In Fig. 2 it is shown that the LA strain has more binding sites than F-22, and that perhaps this is the basis for resistance. The evidence that resistance to emetine can be related to binding sites is fortuitously provided by the LA strain grown at room temperature. These amebas are much more resistant to emetine than those of the same strain which are grown and tested at 37°C, a property that was first reported by Alback and Shaffer. The end-point, i.e. the minimal concentration to produce 100% killing, for 37°C-grown LA is 2×10^{-5}. Those grown at room temperature survive a concentration of 10^{-3} gms/ml, which is the highest we care to go. When tested for H^3-emetine binding, the results are striking. There appears to be a

correspondence between resistance to emetine and total number of binding sites. In Fig. 2 - insert B the LA - 37° curve of A is redrawn to a different scale to allow comparison with the room temperature-grown LA. One could postulate that the change from 37° to room temperature is accompanied by certain structural changes in ribosomes exposing a large number of nonspecific binding sites that would have nothing to do with protein synthesis. It would certainly seem that resistance to emetine in E. histolytica is based on the number of such binding sites.

MATERIALS AND METHODS

Amebas were grown on a modified Schaffer-Frye medium. The LA (Laredo) and F-22 (regular) strains of E. histolytica were used. All tests were conducted in the same medium. To obtain sufficiently large numbers for each experiment amebas were grown in 125 ml. screw-capped Erlenmeyer flasks. The size of the inoculum was adjusted so that the amebas were still in log phase at 48 hours and well into stationary phase at 72 hours. The tests were carried out in 15 ml. screw-capped tubes containing fresh medium, labeled constituents, drug where specified and amebas, in a final volume of 13.0 ml. To each tube were added, depending on the total yield, 3×10^5 to 10^6 amebas. At the end of a period of incubation, duplicate counts of amebas in each tube were made in a hemacytometer. Most experiments were carried out in duplicate.

Protein synthesis was measured as incorporation of radioactive leucine-C^{14} after 3 hrs. incubation at 37°C into acid-precipitable material. Each tube contained 4 γ of added leucine-C^{14} (S.A. = 1×10^9 CPM/ μ mole. Except for those involving puromycin, in emetine binding experiments the incubation time was 60 min., and the final concentration of H^3-emetine was 1×10^{-5} gms./ml. (S.A. = 5×10^6 CPM/mg). In the puromycin experiments the final concentration of H^3-emetine was 1×10^{-4} gms./ml. (S.A. = 1×10^6 CPM/mg). In the "chase" experiments the specific activity (S.A.) was 5×10^7/mg. At the end of experiments, all tubes were placed in an ice bath where they were kept for population determinations and washings. Amebal counts were made after removal of the bulk of the incubation fluid, the volume removed being accurately measured for each tube. The amebas were then washed 5X with successive additions of 10 ml. of fresh, chilled medium, after low speed centrifugation (1,000 g/10 min.) and removal of supernatant fluid, and they were then suspended in a final volume of 1 ml.

For determination of protein synthesis 0.1 ml. of 1% bovine serum albumen as carrier protein and 2 volumes of 10% TCA were added. The resulting pre-

cipitates were washed 2X with 5 ml. aliquots of ethanol, then dissolved in 1.0 ml. of 0.1 N NaOH, transferred to planchets, dried under hot air, and assayed for radioactivity. For H^3-emetine binding the suspension was brought to 2 ml. and made alkaline by addition of 0.1 to 0.2 ml. of 0.1 N NaOH. The free base was then extracted into 4.0 ml. toluene, and an aliquot of 1 ml. was dried in a planchet and assayed for radioactivity. All radioactive counts were performed in a gas-flow, windowless Geiger counter. So that direct comparisons could be made, all activities of incorporated or bound material are expressed as CPM (counts per minute) per 10^5 amebas.

LEGENDS TO FIGURES

Fig. 1. Rate of H^3-emetine binding at 37°C. H^3-emetine conc. = 1×10^{-5} gms/ml. (S.A. = 5×10^8/mg).

■ Strain LA ● Strain F-22

Fig. 2. H^3-emetine binding as a function of concentration - and temperature (LA).

A. Comparison of LA and F-22 strains grown and tested at 37°C. Incubation time was 60 min.

B. Comparison of strain LA grown and tested at 37°C to LA grown and tested at 22°C. Incubation time for latter was 90 min.

Tab. 1. Amebas incubated 1 hr. at 37°C in presence of H^3-emetine (10^{-5} gms/ml) (5×10^6 CPM/mg). Then washed 5 times and for labeled acid precipitable material. All incubations were carried out in duplicate. The above is the average of the calculated results.

TABLE I. Comparison of Protein Synthesis and Sensitivity between "Log Phase" and "Stationary Phase" E. histolytica.

		"Log Phase"			Stat. Phase	
		Concentration of Emetine (gms/ml)	Leucine-C^{14} Incorporated (CPM/10^5 Amebas)	% Inhibition	Leucine-C^{14} Incorporated (CPM/10^5 Amebas)	% Inhibition
Exp.	A	Strain F-22				
		0	35.7	0	23	0
		1×10^{-4}	6.35	82.3	3.0	93
		1×10^{-5}	7.8	79.4	5.0	78.3
		1×10^{-6}	32.0	11.0	23	0
	B	Strain F-22				
		0	80	0	0	0
		1×10^{-4}	6.72	91.7	0	0
		1×10^{-5}	8.9	89.0	0	0
		1×10^{-6}	54.6	32.5	0	0
	C	Strain LA				
		0	29.4	0	19.5	0
		1×10^{-4}	2.9	91.2	1.92	89.7
		1×10^{-5}	10.0	66	3.38	82.3
		1×10^{-6}	23.1	22	19.8	0

TABLE 2. Comparison of H^3-emetine Binding Between "Log Phase" and "Stationary Phase" E. histolytica and Effect of Drugs.

		Drug Added (Final Conc.) (gms/ml)	Log Phase H^3-emetine bound CPM/10^5 amebas	Stat. Phase H^3-emetine bound CPM/10^5 amebas
	Strain			
A.	LA	None	734	915
		Dinitrophenol (10^{-4})	534	742
		Chloroquine (10^{-4})	645	495
		Acriflavin (10^{-4})	834	845
B.	F-22	None	785	581
		Dinitrophenol (10^{-4})	965	845
		Chloroquine (10^{-4})	481	706
		Acriflavin (10^{-4})	515	722

Amebas incubated 1 hr at 37° in presence of H^3-emetine (10^{-5}) 5 X 10^6 CPM/mg.

Then washed (5X), and bound H^3-emetine extracted into toluene.

TABLE 3. Effect of Puromycin on H^3-emetine Binding in LA strain

	Additions (gms/ml)	H^3-emetine bound CPM/10^5 amebas
1.	None	112
2.	Puromycin (5 X 10^{-5}) - Preincubation 1 hr.	108
3.	Puromycin (5 X 10^{-5}) - No preincubation	138

The amebas were incubated for 1 hr with 1 X 10^{-4} gms/ml H^3-emetine (1 X 10^6 CPM/mg). Then washed 5X, and the bound H^3-emetine extracted into toluene following the addition to the final suspension (1 ml) of 0.1 ml of 0.1 N NaOH. In one set of tubes (#2), the amebas were first preincubated with puromycin prior to addition of H^3-emetine. All incubations were carried out in duplicate, and the above are the calculated average results of the two.

TABLE 4. Effect of Puromycin and EDTA on H^3-emetine Binding in LA strain

	Additions (gms/ml)	H^3-emetine bound (CPM/10^5 amebas)
Expt. I	1. None	227
	2. EDTA (1×10^{-4})	184
	3. EDTA (1×10^{-4}) + Puromycin (1×10^{-4})	120
Expt. II	1. None	115
	2. EDTA (3×10^{-4})	82
	3. EDTA (3×10^{-4}) + Puromycin (2×10^{-4})	45

With exception of "additions" conditions were the same as for Table 3. The results are the average obtained from duplicate sets of experiments.

TABLE 5. Exchange (chase) of externally added emetine with bound H^3-emetine and H^3-isoemetine.

A. H^3-emetine bound*

	Before "chase" CPM/10^5 amebas
Expt. 1.	5,320
2.	5,200
3.	5,200
	After "chase" 40X (4×10^{-4}) "cold" emetine 1 hr.
4.	5,840
5.	4,280
6.	6,640

*Inc: 1×10^{-5} H^3-emetine (S.A. = 5×10^7/mg) for 90 min. 37°. Washed 3X, added 4×10^{-4} cold emetine 1 hr. Washed 2X, tested for bound H^3-emetine by means of extraction into toluene. All concentrations are expressed as gms/ml.

B. H^3-isoemetine bound

	Before "chase" CPM/10^5 amebas	
1.	9,280	
2.	10,500	
	After "chase"	% "chased" out
3.	538	94.5
4.	364	96.3

Conditions the same as for A.

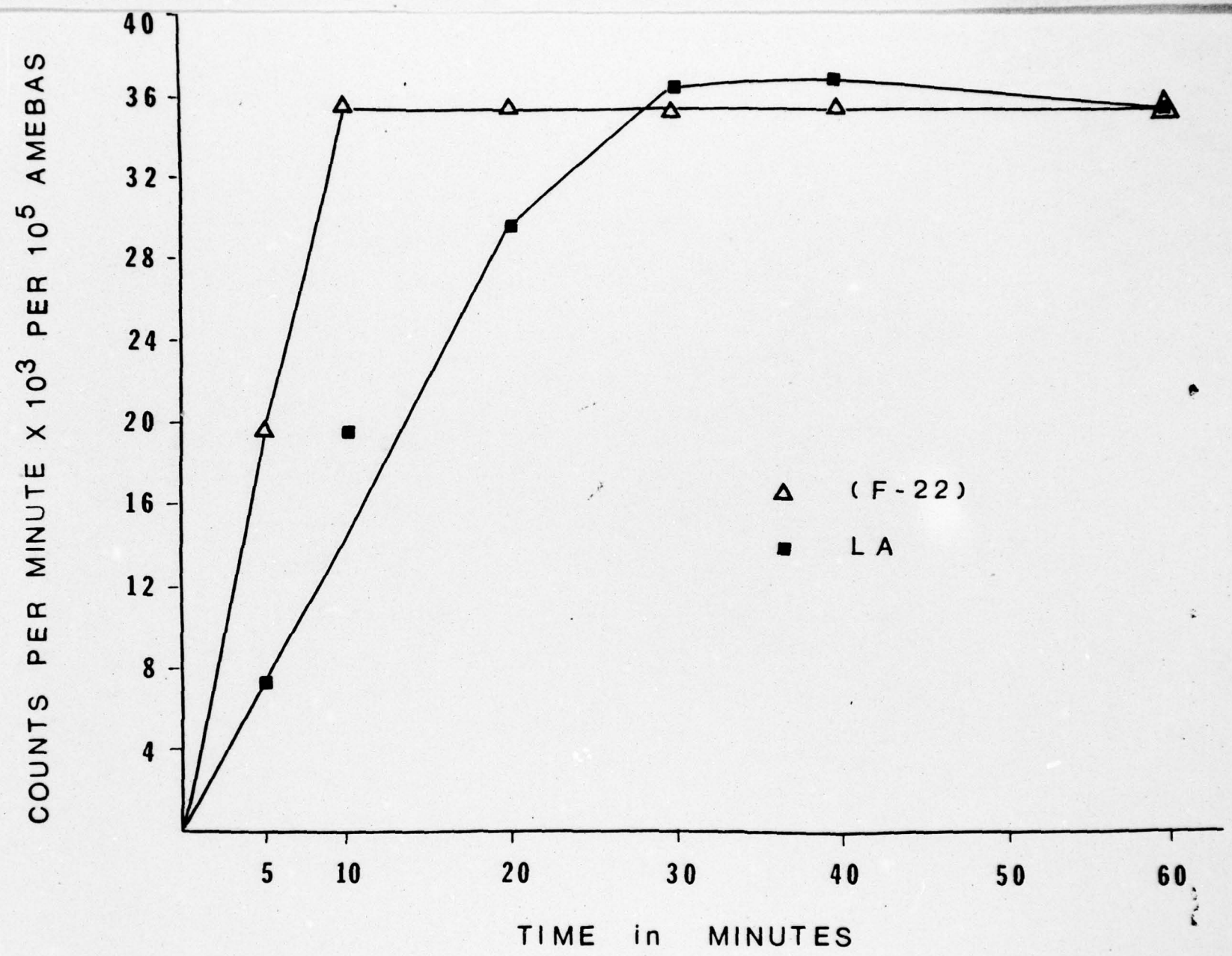
COUNTS PER MINUTE X 10^3 PER 10^5 AMEBAS
40
36
32
28
24
20
16
12
8
4
5
10
20
30
40
50
60
TIME in MINUTES
△ (F-22)
■ LA

FIG. 1.

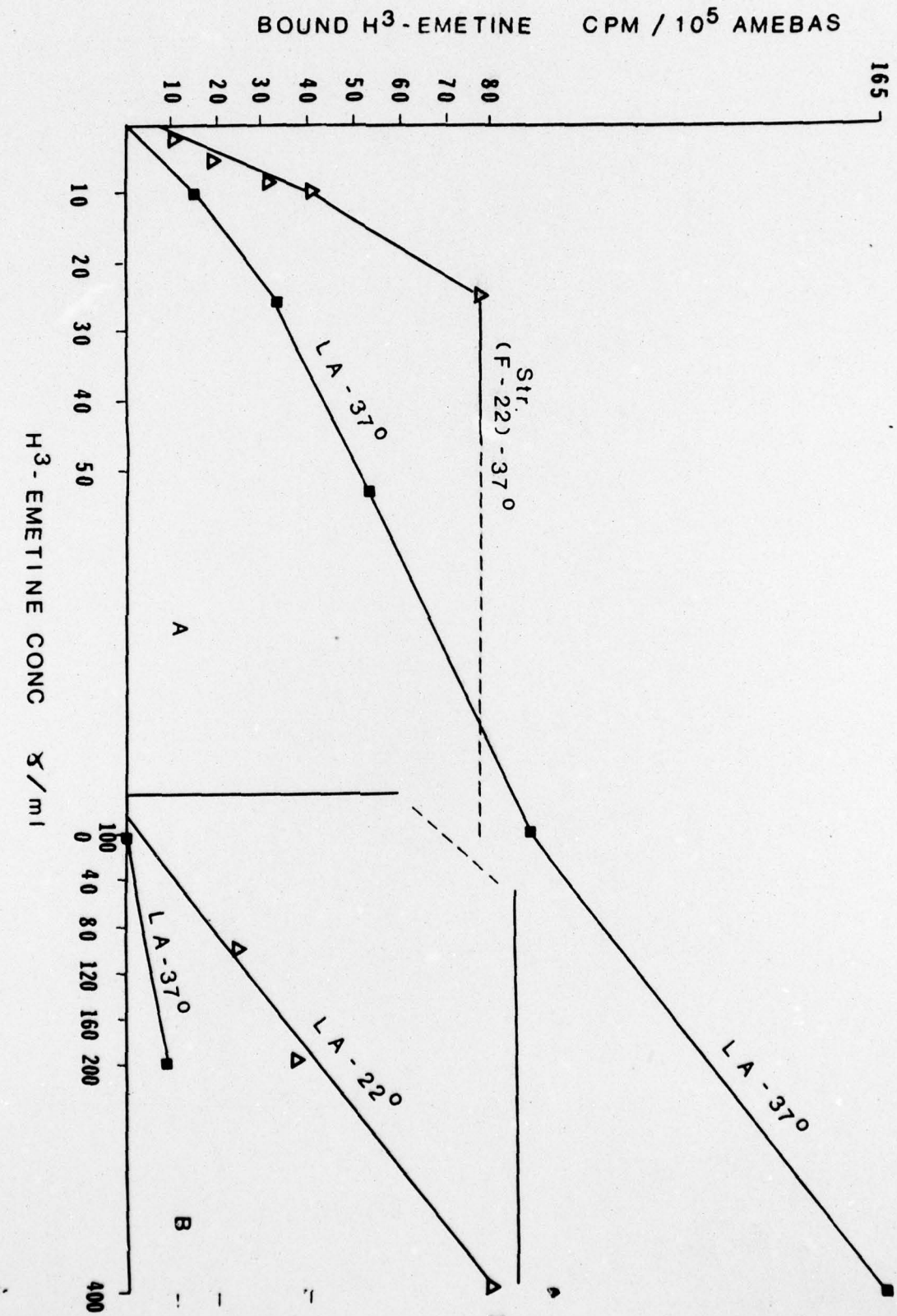
BOUND H3 - EMETINE CPM / 10^5 AMEBAS
165
10
20
30
40
50
60
70
80
Str. (F-22) - 37°
LA - 37°
A
H3 - EMETINE CONC ϰ/ml
10 20 30 40 50
100
0 40 80 120 160 200
400
LA - 37°
LA - 22°
B
1600
800
400
200

Fig. 2.

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