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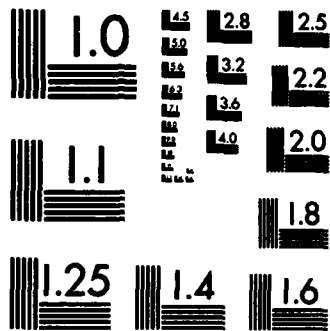
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THE EFFECTS OF SPECIFIC ENVIRONMENTAL POLLUTANTS ON THE
BIOSYNTHETIC FUNCTIONS OF MAMMALIAN CELLS IN VITRO:
A SEARCH FOR STRUCTURE/ACTIVITY RELATIONSHIPS

Annual and Final Report

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) A rabbit reticulocyte cell-free translation system was used to evaluate the effects of specific environmental pollutants on mammalian protein (globin) synthesis. Dose-response relationships were determined over a range from three to eight logs of concentration (generally 10^{-12} to 10^{-6} M) for the following benzene and toluene derivatives: 1) benzene and various chloro- and nitro -substituted benzenes; 2) assorted chloro, mononitro-, and monoamine-substituted toluenes. 3) all the dinitrotoluenes and,		

(#20)

4) 2,4,6 trinitrotoluene. The results indicate that no significant inhibition of protein synthesis occurs at concentrations below 10^{-5} M for the majority of the agents tested. Solubility problems hindered testing at higher concentrations although in cases testing up to the millimolar range was possible. Four of the compounds tested showed inverse relationships with greater inhibition at lower concentrations. Only one agent, 2,3 dinitrotoluene, displayed typical concentration-dependent inhibition but that only at concentrations greater than 10^{-5} M. Inhibitory effects were observed for: 4-amino-3,5 dinitrotoluene (15-35% for 10^{-6} - 10^{-12} M); hexachlorobenzene (10-35% for 10^{-7} - 10^{-12} M); 4-nitrotoluene (15-50% for 10^{-5} - 10^{-12} M); nitrobenzene (5-30% for 10^{-6} - 10^{-12} M) and 2,3 dinitrotoluene (10-25% for 10^{-5} - 10^{-3} M). The translation bioassay method is relatively simple and quick and shows good promise as a general procedure for evaluating chemical agents for toxicity.

SUMMARY

This report summarizes the results obtained for the tenure of the two-year contract, 1 August 1977 - 31 July 1979.

Summary of Results

The rabbit reticulocyte cell-free translation system for studying globin biosynthesis was established and optimized for individual reaction components. The system was shown to respond normally to well established inhibitors of protein synthesis; i.e. it is sensitive to cycloheximide and puromycin, but resistant to chloramphenicol, which is specific for prokaryotic protein synthesis. Using this system, we have evaluated the effects on translation of: 1) benzene and various chloro- and nitro-substituted benzenes (specifically hexachlorobenzene, nitrobenzene and 1-chloro, 2-nitrobenzene; 2) various chloro-, mononitro-, and monoamino, dinitro-substituted toluenes (specifically orthochlorotoluene, metachlorotoluene, parachlorotoluene, 4-nitrotoluene, 2-amino-4-nitrotoluene, 4-amino-2,6-dinitrotoluene and 4-amino-3,5-dinitrotoluene); 3) all the dinitrotoluenes and 4) 2,4,6-trinitrotoluene. The effects of several potential solubilizing vehicles for delivery of those agents to the assay system were evaluated at the onset of the study. These included: methanol, ethanol, ethylene glycol, diethyl ether, acetone and dioxane; the detergents Tween-20; and mixes of Tween-20 and methanol or dioxane. Based on these latter tests 1% ethanol was selected as the common solvent for the assorted test agents; at this level no positive or negative effects were evident.

For each of the test compounds dose-response relationships were determined over a range of three to eight logs of concentration, generally 10^{-12} to 10^{-6} M; in most cases, solubility problems hindered attempts to assess effects at higher concentrations.

For most of the compounds evaluated no inhibitory effects were evident at concentrations below 10^{-5} M. Four of the agents tested showed surprising inverse relationships with greater inhibition at lower concentrations. Only one agent, 2,3-dinitrotoluene displayed typical concentration-dependent inhibition, but that only at concentrations in excess of 10^{-5} M.

The extents of inhibition and concentration levels at which inhibitory effects were observed were:

- 1) 4-amino-3,5-dinitrotoluene (15-35% for 10^{-6} - 10^{-12} M),
- 2) hexachlorobenzene (10-35% for 10^{-7} - 10^{-12} M),
- 3) 4-nitrotoluene (15-50% for 10^{-5} - 10^{-12} M),
- 4) nitrobenzene (5-30% for 10^{-6} - 10^{-12} M), and
- 5) 2,3-dinitrotoluene (10-25% for 10^{-5} - 10^{-3} M).

When all the results are taken together no obvious structure-function relationships were evident.

Studies were also initiated to test the effect of the di- and tri-nitrotoluenes on whole red blood cell translation using intact rabbit reticulocytes. No significant inhibition was obtained with 2,3- or 2,4-dinitrotoluene, at 10^{-4} M whereas 2,4,6-trinitrotoluene at 10^{-5} M caused approximately 30% inhibition. Additional studies are needed to further substantiate these last findings.

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Introduction

A multipart research program was developed to yield information about the potential for hematologic toxicity for specific benzene and toluene derivatives. The effects of these agents were evaluated using bioassays for erythroid cell proliferation and heme- and globin synthesis. Testing was carried out by personnel in two separate divisions at the W.R.A.I.R. (Divisions of Medicine and Medicinal Chemistry) and two groups at Georgetown University and the University of Massachusetts. The overall program was initiated, and coordinated by LTC Michael J. Haut, then Chief, Dept. Hematology at W.R.A.I.R. Col. Leslie B. Atstatt, Director, Division of Medicine at W.R.A.I.R. shared planning and coordination responsibilities with Haut.

The list of agents to be evaluated was developed by Dr. Haut in consultation with toxicologists at Fort Detrick. The compounds selected included known and potential pollutants being introduced into the environment by various army installations. This report summarizes the findings of the University of Massachusetts group relating to effects on protein biosynthesis.

The cell-free synthesis of protein was selected as one of several prototype systems to use in examining the effects of toxic agents. Because the mechanism of globin synthesis is one of the most thoroughly understood it was deemed an excellent model system both for screening potential toxins for effects on translation and for analyzing mechanisms of action where inhibitory effects are observed.

The overall objectives of the program were (1) to establish an in vitro cell-free assay system to evaluate the effects of specific environmental pollutants on mammalian protein biosynthesis; (2) to determine if structure-activity relationships exist which would make it possible to predict relative toxicities of untested compounds on the protein biosynthetic process; (3) to eventually determine the subcellular site(s) of action of model pollutants which inhibit mammalian translation. Only the first two objectives were addressed in the period for which support was received. A suitable bioassay method was established and some 18 agents tested. The majority of the compounds had little effect on translation at concentrations below 10^{-5} M, the level at which complete solubility was believed to obtain. Of the agents showing positive or negative effects no striking structure-function relationships were observed.

Methods and Materials

Preparation of red blood cells and lysates--Reticulocytes were obtained from New Zealand white rabbits after induction of reticulocytosis by phenylhydrazine injection, essentially as described by Crystal et al. (Crystal, R.G., Elson, N.A., and Anderson, W.F. Initiation of globin synthesis: Assays. Chapter 12 in Methods in Enzymology XXX. Nucleic Acids and Protein Synthesis. Moldave, K., and Grossman, L., eds., Academic Press, NY, 1974.) Rabbits were bled by cardiac puncture and the reticulocyte-enriched (75-90%) red blood cells collected by differential centrifugation and freed of plasma by washing in buffer. Except where assays were done with intact cells membrane-free lysates were prepared by osmotic shock and centrifugation at $20,000 \times g$. Lysates prepared by this method can be stored at -85°C for at least one year with no significant loss in ability to synthesize globin.

Protein synthesis--Globin synthesis was determined by measuring the incorporation of ^3H -leucine into acid-insoluble protein. The procedures used were those described by Woodard *et al.* (Woodard, W.R., Ivey, J.L., and Herbert, E., Protein synthesis with rabbit reticulocyte preparations. Chapter 67a in *Methods in Enzymology XXX. Nucleic Acids and Protein Synthesis*. Moldave, K., and Grossman, L., eds., Academic Press, NY, 1974). The optimum concentrations of each of the reaction components was determined empirically in preliminary assays. The results of these optimization assays are shown below.

The benzene and toluene derivatives tested have low solubilities in water. For this reason dose response measurements were carried out over a concentration range that often did not exceed 10^{-5} M. In cases it was possible to extend the range to 10^{-4} M. Solubilities were determined empirically by visual examination of a dilution series. This was necessary because of the lack of published solubility values. The point of maximum solubility was considered to be the highest concentration at which sample clarity was maintained with only a single phase in evidence.

Results and Discussion

Evaluation of the cell-free system and optimization--In verification studies, it was determined that an increase in ionic strength in the assay system resulted in an increase in protein synthesis of approximately 100%. Since the increase in ionic strength might also change the concentration optimum for other assay components, the system was reoptimized for all individual components. The final concentrations of assay components in each of the following figures are as follows unless otherwise designated in the figures. Lysate, 40 μl /100 μl assay; creatine phosphate, 0.011 M; KCl, 0.11 M; MgCl_2 , 0.53 mM; creatine phosphokinase, 0.04 mg/ml; unlabelled amino acids, 0.11 mM each; ^3H -leucine, 0.044 mM (5 Ci/mmmole). All assays both for optimizing the system and subsequent testing of pollutants have been carried out at least in triplicate and in some cases in quadruplicate. Analysis of data points from 68 independent assays using 8 independent preparations of lysate show the variance due to experimental error to be less than $\pm 8\%$. The optimized systems typically show incorporation of 0.14 - 0.189 μmoles of ^3H -leucine into acid precipitable protein in 40 min.

Optimization for reaction components--The results in Figure 1 show the incorporation activity for the smallest amount of lysate (40 μl /100 μl assay) which gives an essentially linear response with respect to time and at the same time results in levels of ^3H -leucine incorporation that are well above zero time (background) radioactivity. Figure 2 demonstrates that 0.011 M creatine phosphate is optimal for this assay constituent and results in virtually linear incorporation of ^3H -leucine with respect to time. The results in Figure 3 demonstrate that 0.08 mg/ml of creatine phosphokinase may be somewhat more optimal for protein synthesis than 0.04 mg/ml at longer time points.

That ATP cannot replace creatine phosphate as an energy source in our hands (either at equimolar or higher concentrations) is shown in Figure 4. Indeed, the higher the ATP concentration, the lower the protein synthesis activity. Furthermore, addition of ATP to the assay system containing creatine phosphate as energy source inhibited activity more than 93%. To test whether the higher concentrations of ATP were chelating Mg^{++} which may be needed in a free form, various ratios of ATP:Mg were tested.

Figure 5 shows that increasing Mg^{++} does improve protein synthesis with ATP as energy source, at least up to 1:1 ratio of Mg^{++} :ATP. Above this ratio, Mg^{++} is inhibitory. Also, as shown, increasing the Mg^{++} concentration is inhibitory when creatine phosphate is the energy source. $MgCl_2$ and creatine phosphate concentrations of 0.53 mM and 0.01 M, respectively were optimal. Figure 6 reveals the optimum concentration of KCl to be 0.11 M. Again, increasing Mg^{++} above 0.53 mM is inhibitory.

The optimum concentrations of the assay components as determined are given in Table 1. These concentrations were used in all subsequent assays.

Response to inhibitors--The assay system was next tested for its response to well established inhibitors of protein synthesis. As shown in Figure 7, cycloheximide, which is specific for eukaryotic ribosomes, inhibits 3H -leucine incorporation some 68%, 90% and 95% at concentrations of 10^{-5} M, 10^{-4} M and 10^{-3} M, respectively. Similarly, puromycin, which will inhibit both eukaryotic or prokaryotic protein synthesis inhibits some 79% and 99% at concentrations of 10^{-5} M and 10^{-4} or 10^{-3} M, respectively (Figure 8). On the other hand, chloramphenicol, which is specific for prokaryotic or mitochondrial ribosomes shows little inhibition of this eukaryotic system. Figure 9 indicates chloramphenicol only inhibits 3%, 15% and 19% even at the high concentrations of 0.5×10^{-3} M, 1.0×10^{-3} M, and 1.5×10^{-3} M, respectively. These results are in good agreement with those generally observed and verify that the reticulocyte lysate cell-free system is performing normally.

Examination of solubilizing vehicles for delivery of the benzene and toluene derivatives--Because the WRAIR groups initially used methanol for solubilizing porphyrin in the heme synthetase reaction, and Tween-20 in preparing homogenates for both the alpha-levulinic acid synthetase and heme synthetase reactions, a survey of the effects of these solubilizers on translation was carried out early in the first year.

The effects of six non-aromatic solvents, one detergent, and two mixtures of solvent and detergent were tested in the cell-free globin translation system. The solvents analyzed included: methanol, ethanol, ethylene glycol, diethyl ether, acetone and dioxane; the detergent was Tween-20 and the mixes of detergent and solvent were Tween-20 plus methanol or dioxane. The results of these assays are shown as dose-response curves in Figures 10 and 11 and in tabular form in order of decreasing potency in Table II.

Not surprising, the solvents (and detergent) tested varied greatly in their effects on translation. In order of increasing potency the relative toxicities of the simple solvents (over the concentration range of 0-4%) was determined to be: diethyl ether, ethylene glycol, ethanol, dioxane, acetone and methanol. Effects on protein synthesis ranged from 10% stimulation by traces (.02%) of ether to 85% inhibition by methanol at concentrations in excess of 1%. For all but methanol there was little effect ($\leq 10\%$) on translation at the 1% concentration level; at concentrations of 4%, inhibition varied from 20-60% of control values. The detergent, Tween-20, had essentially no effect on protein synthesis over the range of 0-4%; the presence of Tween-20 neutralized somewhat the inhibitory effects of methanol and dioxane.

In the end, each of the research groups agreed to use 1% ethanol as solubilizer so that results from the individual studies could better be correlated.

Effects of benzene and derivatives--The results shown in Figures 12 and 13 indicate 12%, 13% and 10% stimulation of protein synthesis by benzene after 45 min at concentrations of 2×10^{-4} M, 2×10^{-5} M and 2×10^{-6} M, respectively. Figure 14 reveals the effect of 1-chloro-2-nitrobenzene on protein synthesis. Little if any effect ($\leq 10\%$) was observed for concentrations of 10^{-5} M and 10^{-12} M.

Nitrobenzene (Figure 15) appeared to show an inverse dose-response effect. Inhibition increased from 10% to 30% between 10^{-6} M and 10^{-12} M. Further studies are needed to clarify this puzzling result.

The effect of hexachlorobenzene on globin synthesis is shown in Figure 16. Although the triplicate points in each of these two experiments agree within 5%, the two experiments gave quantitatively different results. In each case, however, inhibition seemed to be greater at lower concentrations of agent, at least between concentrations of 10^{-9} M and 10^{-12} M. This inverse dose-response curve is also unexpected and not yet understood.

Effect of chloro-, mononitro-, and monoamino dinitro-substituted toluenes--Figure 17 shows the effect of o-chlorotoluene on globin formation. While results from experiments 1 and 2 agree well at lower concentrations of agent there are significant differences at the higher concentrations of 10^{-7} M and 10^{-8} M. The basis for the disparity are not clear as results of quadruplicate assays revealed variation of only $\pm 6\%$ for a given assay. In any case, no significant inhibition of globin synthesis was observed by this agent. Similarly, m-chlorotoluene (Figure 18) and p-chlorotoluene (Figure 19) appeared not to cause significant inhibition although in the latter case, higher concentrations of p-chlorotoluene (10^{-7} , 10^{-8} M) inhibited up to 27% in one experiment.

The effect of 4-nitrotoluene is given in Figure 20. The results of quadruplicate assays show variation of only $\pm 6\%$ in a given experiment. Consistent results were obtained between two independent experiments at all concentrations of agent except 10^{-8} M and 10^{-9} M where variation of 30% and 20%, respectively were seen. Like hexachlorobenzene, this agent seemed to show an inverse dose-response relationship.

The effects of 2-amino-4-nitrotoluene and 4-amino-2,6-dinitrotoluene are shown in Figures 21 and 22, respectively. No significant inhibition was seen with either of these agents over the concentration range of 10^{-12} - 10^{-6} M.

The effect of 4-amino-3,5-dinitrotoluene, shown in Figure 23, is a low, but consistent inhibition of 15 to 35% (10^{-12} - 10^{-6} M) with a trend of greater inhibition at lower concentrations.

Effect of the dinitrotoluenes--At a joint meeting of all investigators held at WRAIR in December, 1978, it was decided to give high priority to studies on the dinitrotoluenes (DNT). Since earlier studies with 2,3-, 2,4-, 2,5-, and 3,4-DNT had shown little, if any, inhibition of globin synthesis, we decided to perform kinetic studies instead of single 40 min time points to more fully substantiate the apparent absence of an inhibitory effect. The DNT's and concentrations tested were the 2,3-,

2,4-, and 3,4- isomers at 10^{-3} - 10^{-5} M; 3,5-DNT at 10^{-4} - 10^{-6} M and 2,6-DNT at 10^{-4} - 10^{-6} M.

The only dinitrotoluene to show a concentration effect was 2,3-DNT. The results for three experiments are shown in Figures 24A-C and in Table 3. Inhibition effects of 10-30% were observed for 10^{-3} M DNT, but it was not consistent from experiment to experiment. Triplicate samples in each case showed variation of only $\pm 6\%$.

The effects of 2,4-DNT, 2,5-DNT, 2,6-DNT, 3,4-DNT and 3,5-DNT are shown in Figures 25-29 respectively. No significant inhibition of globin synthesis was obtained using any of these agents at the concentrations evaluated. Thus, the dose-response effects obtained in the assays with 2,3-DNT could provide the basis for a structure-function relationship.

Effect of 2,4,6-trinitrotoluene--Due to the solubility of these compounds, only concentrations below 10^{-5} M could be tested. Figure 30 shows the effect of 2,4,6-trinitrotoluene. No significant inhibition by this compound was observed at any concentration after either 20 min or 40 min of incubation.

Studies on intact reticulocytes--Cells were prepared for each experiment but were not lysed. The average of the results of two experiments each performed in triplicate using 2,4,6-trinitrotoluene, 2,4-dinitrotoluene and 2,3-dinitrotoluene are shown in Figure 31. As shown, 2,3-DNT and 2,4-DNT have no significant effect on globin synthesis at 10^{-4} M whereas 2,4,6-trinitrotoluene showed approximately 30% inhibition at 10^{-5} M at both 20 and 40 min. More firm conclusions concerning the effect of this agent in this system await further study.

Specific versus non-specific effects: a caveat about interpretation of the above data--While the basis for the effects of the solubilizers and substituted aromatic agents are not known it is reasonable to suggest that at least some of the effects are non-specific in nature. Most of these agents are known or can be predicted to cause denaturation of biological macromolecules by disrupting the hydrophobic and hydrophilic interactions which stabilize tertiary structure. At low concentrations of a denaturing agent, proteins and nucleic acids such as those involved in translation may still function albeit at reduced efficiency; at higher concentrations activity may be completely abolished with loss of tertiary structure. In the cases of several enzymes it has been demonstrated that partial denaturation can result in increased activity - usually as a result of an alteration in the affinity of the enzyme for a substrate (the Michaelis association constant) rather than in the rate of catalysis.

In those cases where the dose-response plots are "lumpy", i.e., where a direct correlation between concentration and effect is not observed, it may be that the data are reflecting changes in multiple activities with different sensitivities toward the pollutant in question.

It is quite possible of course that some of the effects observed by us reflect specific inhibition. That is, one or more of these solvents or aromatic agents may affect a particular activity or activities as a competitive/non-competitive/uncompetitive inhibitor. Discrimination between specific and non-specific action is only possible through careful kinetic analysis in which only single biochemical reactions are studied.

In the case of translation in a eukaryotic cell at least 120-130 different macromolecules are involved. The list of elements includes: 40-60 unique transfer RNAs, at least 20 aminoacyl-tRNA synthetases; at least four types of ribosomal RNA, 50-55 ribosomal proteins, and at least a half-dozen peptide initiation, elongation and termination factors. To identify the subcellular site of action of these or any other agents it will be necessary to reduce the complexity of the assay system by studying the partial reactions of protein synthesis. For the purpose of cataloging pollutants which are inhibitors of protein synthesis it will most likely be sufficient to determine if the effect(s) is/are at the level of aminoacylation, initiation, elongation or termination.

Conclusions

The cell-free globin assay system constitutes a good bioassay method for evaluating effects of chemical agents on mammalian protein biosynthesis. Assays can be carried out in on several test compounds simultaneously with same day results. The method gives good precision (6-8%) generally although inconsistencies were observed in some cases in the present study; the basis of the disparity is unknown but could be due to spurious effects of the test agent itself.

The bulk of the compounds tested showed no significant inhibitory effects over the concentration range of 10^{-12} to 10^{-5} M. Owing to generally low solubility the concentrations of these compounds in environmental water is not likely to exceed these levels in the worst cases. While the results presented indicate that only a few of the agents tested can be expected to inhibit protein synthesis in a contaminated eukaryotic organism we certainly cannot conclude that the remaining compounds are harmless. Neither can it be assumed that compounds causing negative (or positive) effects will be safe at concentrations below those at which the effects are manifest. For the reasons cited above any number of specific and nonspecific effects could occur which would be deleterious to an organism well before cellular protein synthesis is effected. The importance of interpreting the results in proper context cannot be overemphasized. Where detailed evaluations of potentially toxic agents are needed it will be important to utilize an assortment of bioassay methods based on different physiological functions. The assays used in the current WRAIR-Georgetown-UMass joint study are good ones, i.e. cell proliferation, mitochondrial function and genetic translation. Others that should be considered include mutagenesis, transport, membrane potential, neurotransmission, etc. Such a battery of assay methods will have important predictive value and will markedly reduce the need for whole organism testing.

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Table 1. Optimum Concentrations of Protein Synthesis Assay Components

<u>Component</u>	<u>Concentration</u>
Lysate	40 μ l/100 μ l assay
Creatine phosphate	0.011 M
KCl	0.11 M
MgCl ₂	0.53 mM
Creatine phosphokinase	0.04 mg/ml
Unlabelled amino acids	0.11 mM each
³ H-leucine	0.044 mM (5 Ci/mmmole)

Table 2. Effect of Various Solvents and a Detergent on Cell-Free Synthesis of Rabbit Globin: Relative Activity of Incorporating System as a Function of Solvent/Detergent Concentrations

<u>Solvent</u>	<u>Relative Activity (%)</u>					
	<u>.2%</u>	<u>1%</u>	<u>2%</u>	<u>4%</u>	<u>6%</u>	<u>10%</u>
methanol	-	27	30	16	-	-
acetone	-	-	54	43	31	11
dioxane	-	-	63	48	-	-
dioxane + 2% Tween-20	-	-	75	52	-	-
ethanol	95	96	80	-	-	-
methanol + 2% Tween-20	-	-	93	63	-	-
ethylene glycol	-	-	92	71	48	23
Tween-20	-	-	88	102	-	-
ether	112	100	102	81	-	-

Table 3. Effect of 2,3 Dinitrotoluene on Globin Synthesis

	<u>Inhibition at 40 min (%)</u>		
	<u>10⁻⁵ M DNT</u>	<u>10⁻⁴ M DNT</u>	<u>10⁻³ M DNT</u>
Expt. 1	9	11	24
Expt. 2	1	0	5
Expt. 3	0	3	19

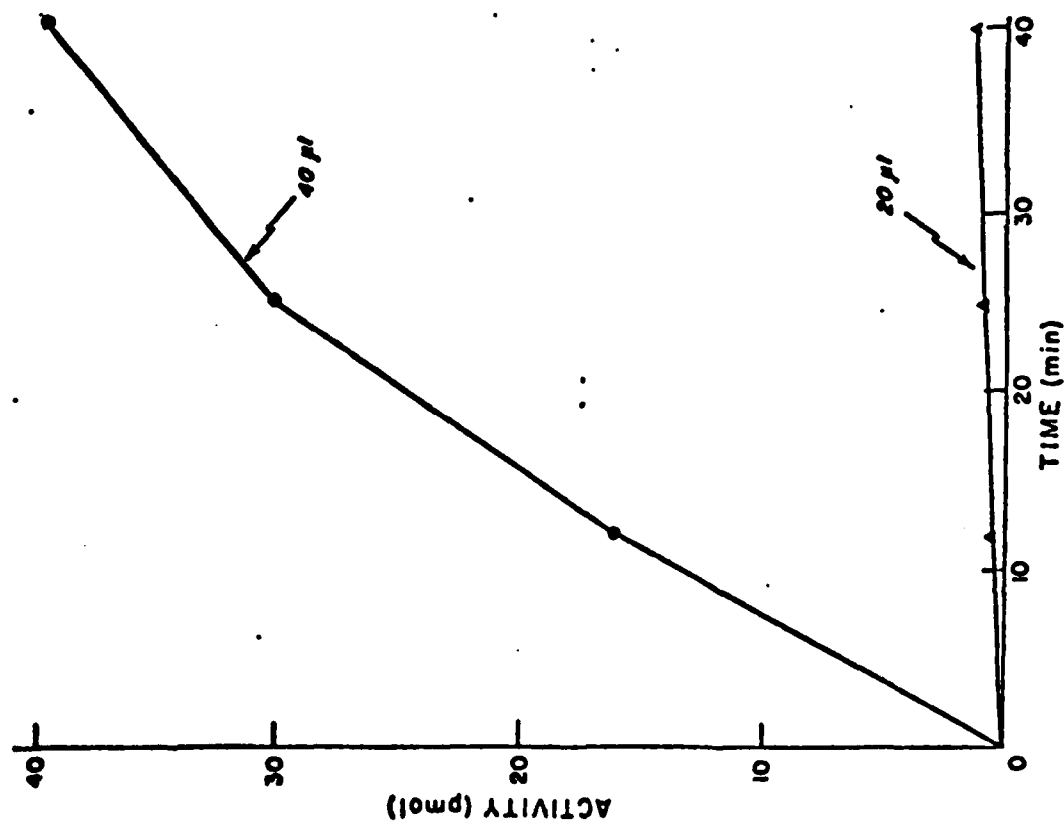


Fig. 1.

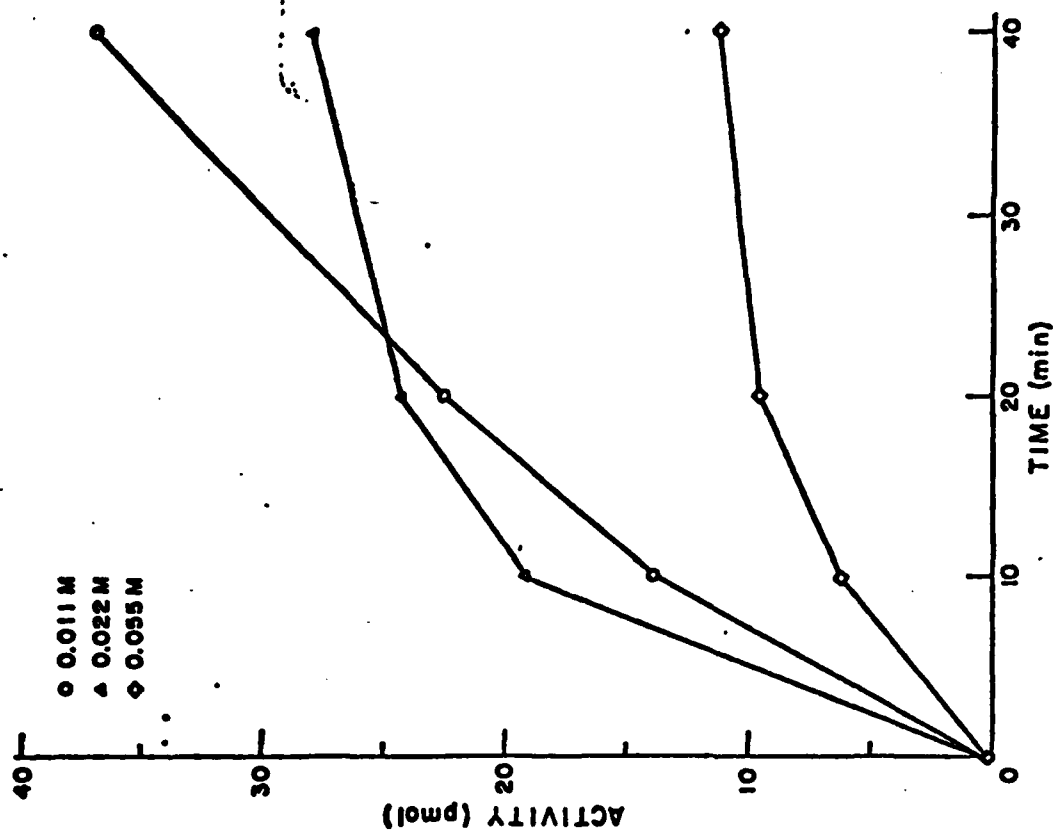


Fig. 2.

Dependence of globin synthesis on lysate (Fig. 1) and creatine phosphate (Fig. 2) concentration.

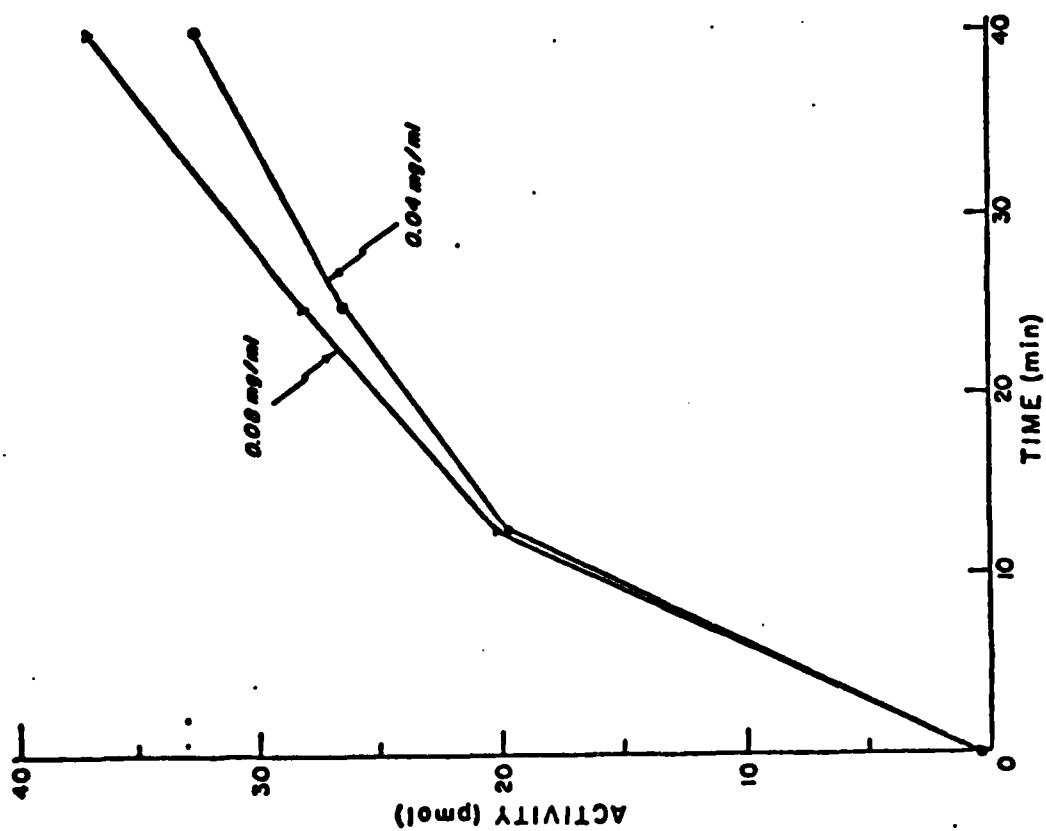


Fig. 3.

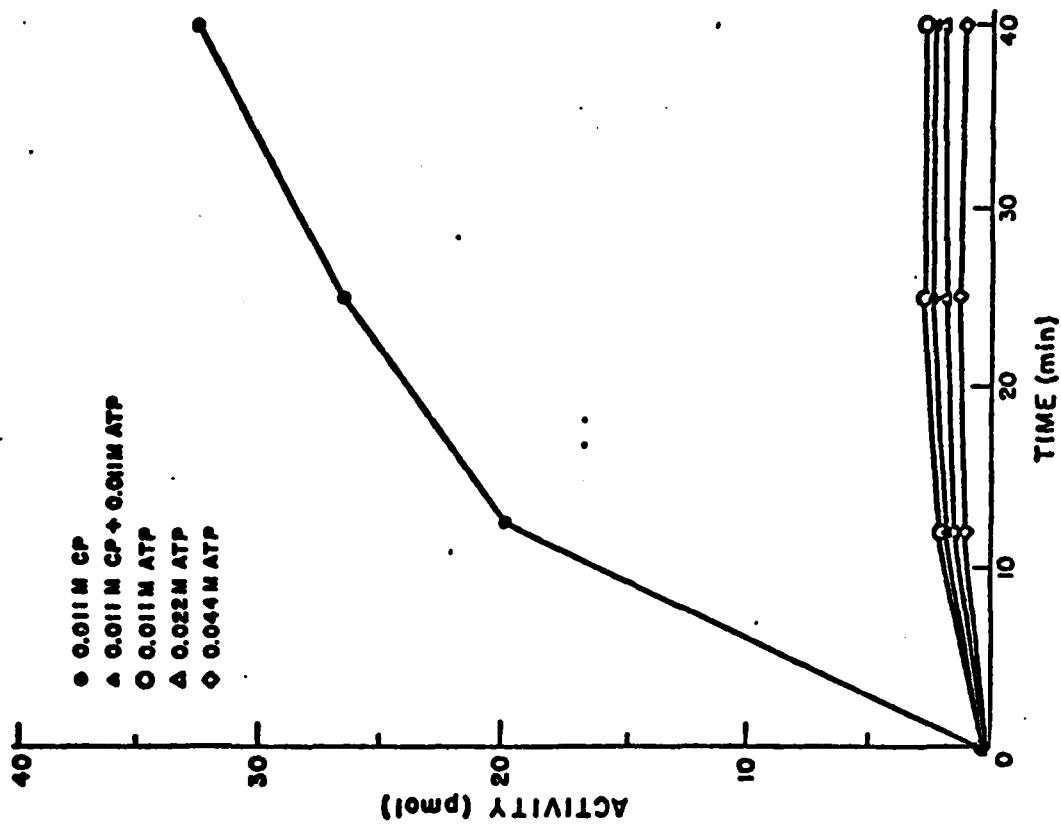


Fig. 4.

Effects of creatine phosphokinase (Fig. 3) and ATP (Fig. 4) levels in globin synthesis.

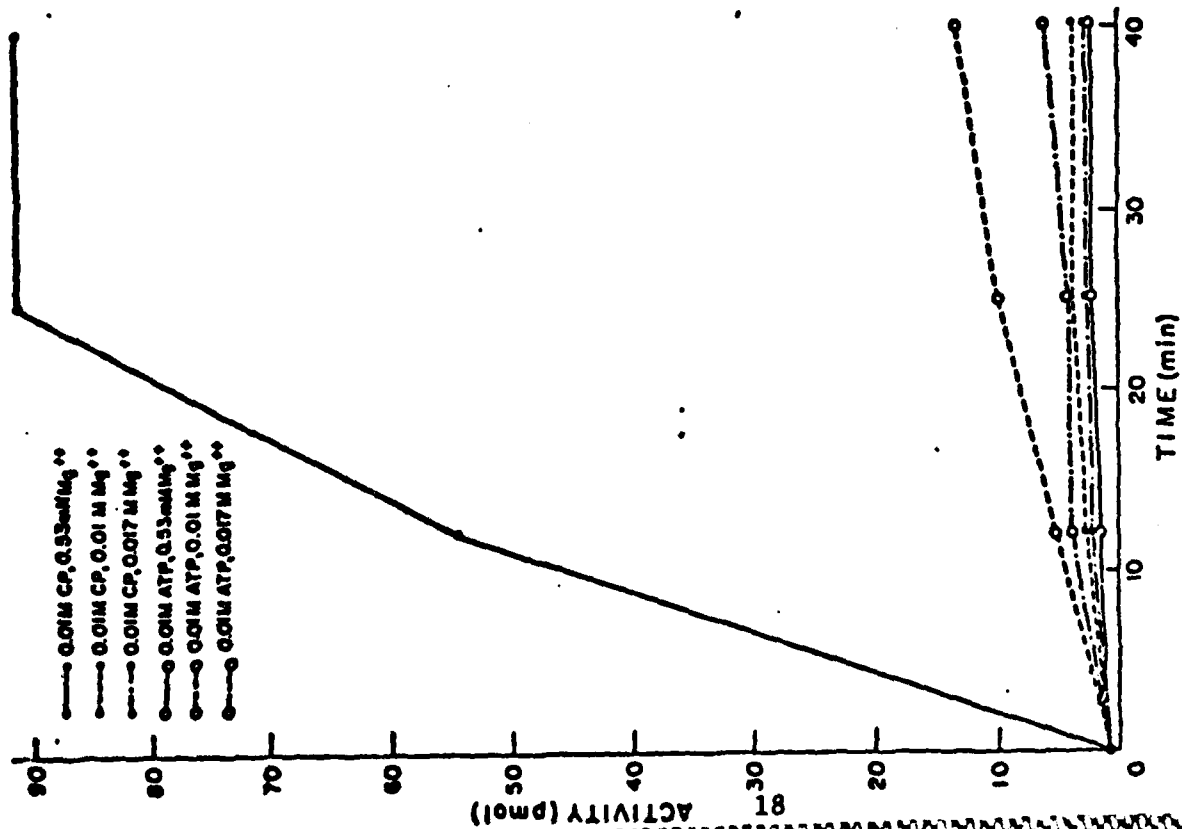


Fig. 5.

Dependence of globin translation on ATP: $MgCl_2$ and creatine phosphate: $MgCl_2$ ratios (Fig. 5) and KCl concentration (Fig. 6).

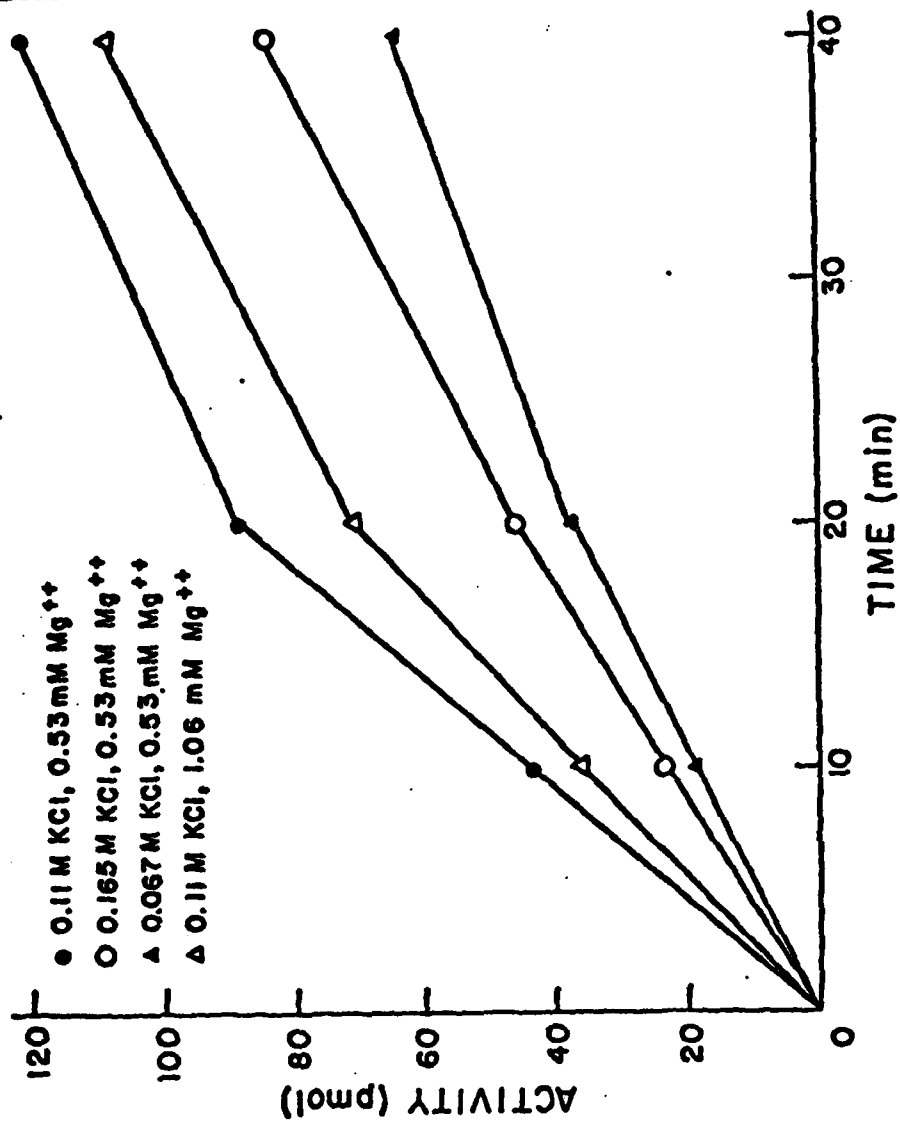


Fig. 6.

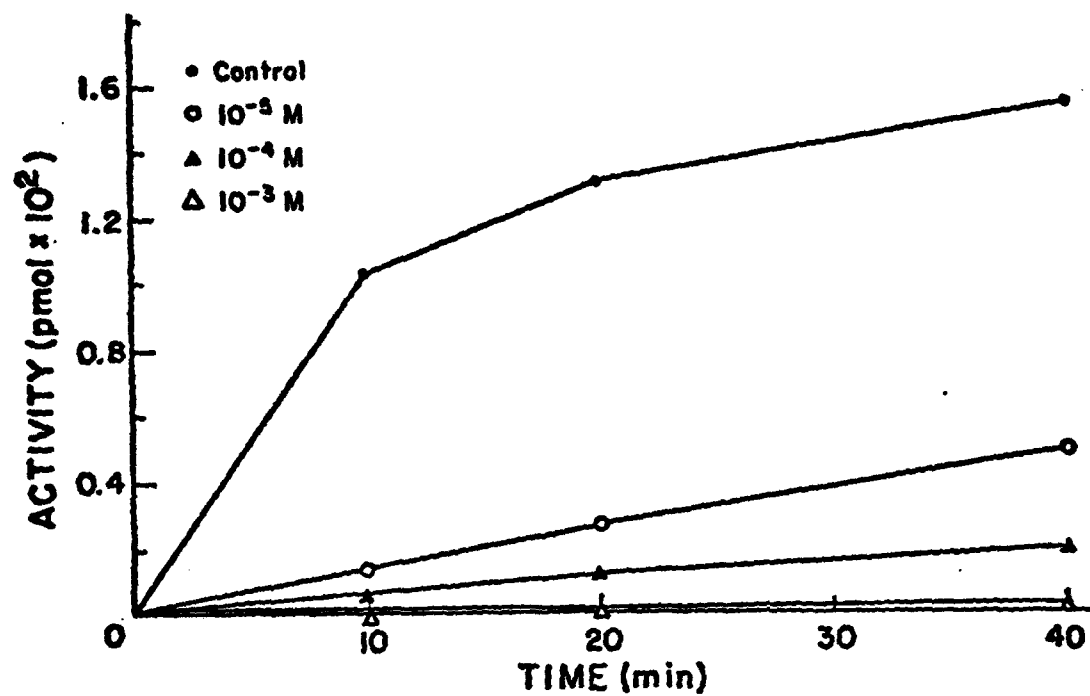


Fig. 7.

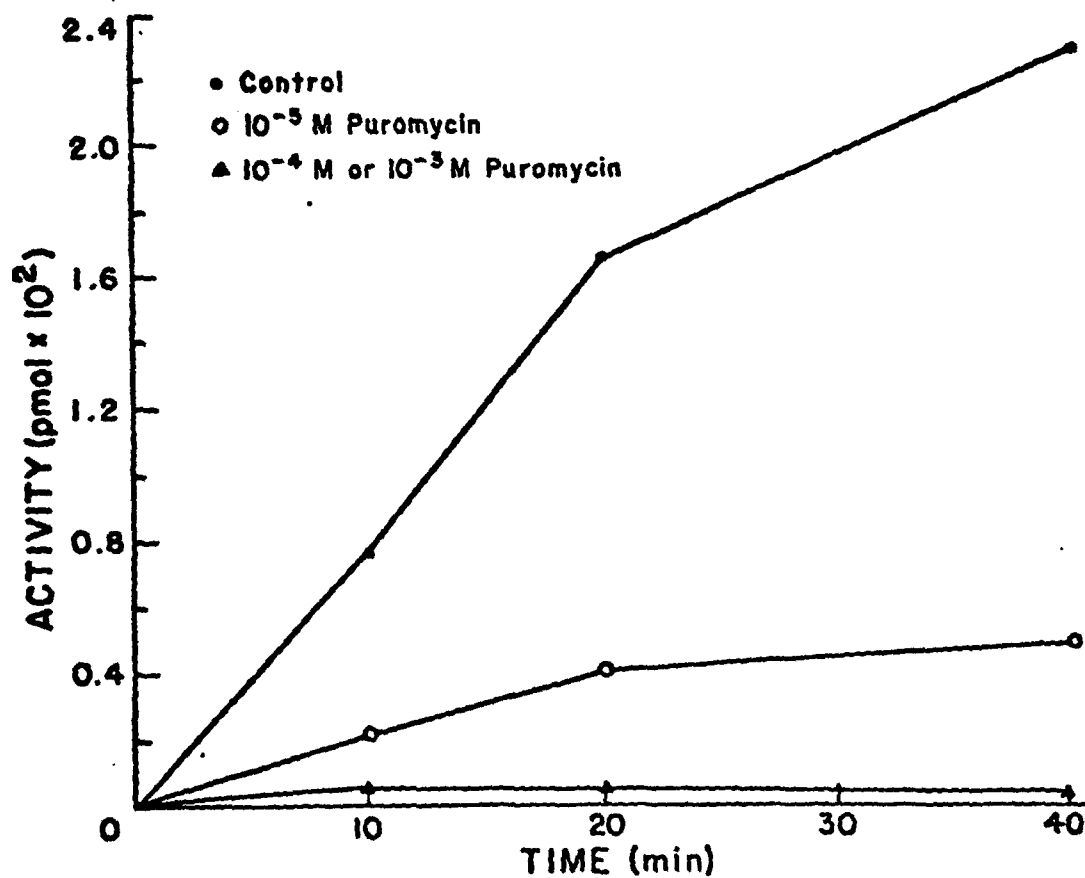


Fig. 8.

Inhibition of globin synthesis by cyclohexamide (Fig. 7) and puromycin (Fig. 8).

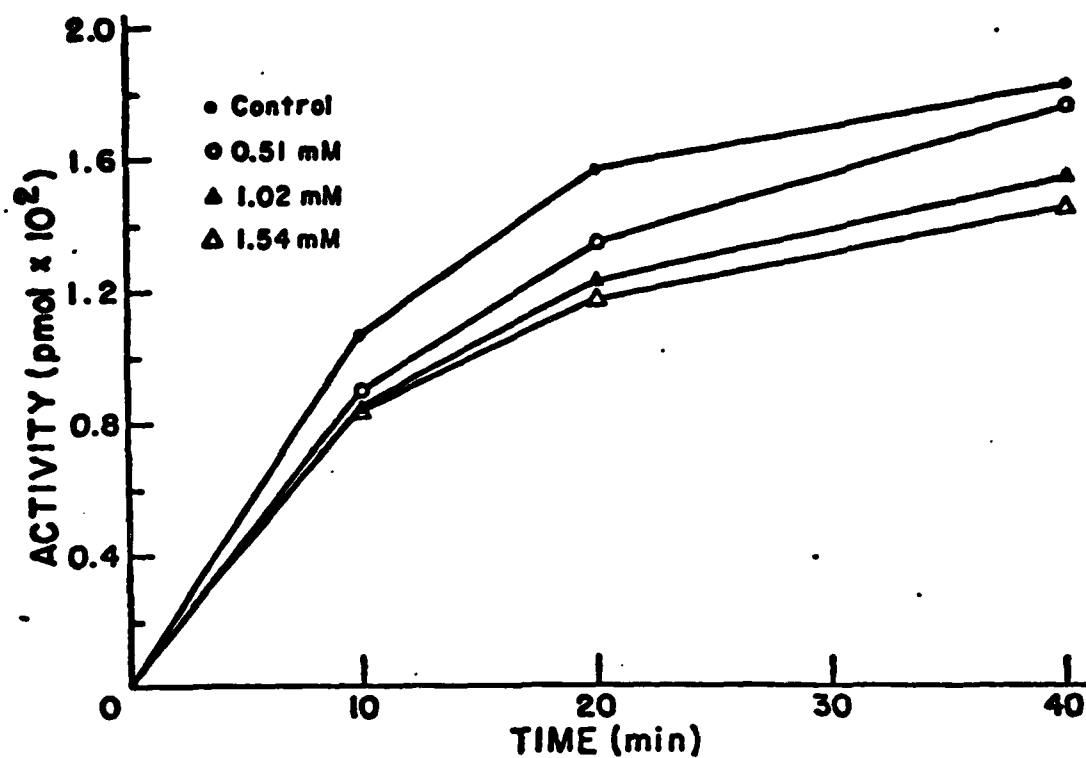


Fig. 9.

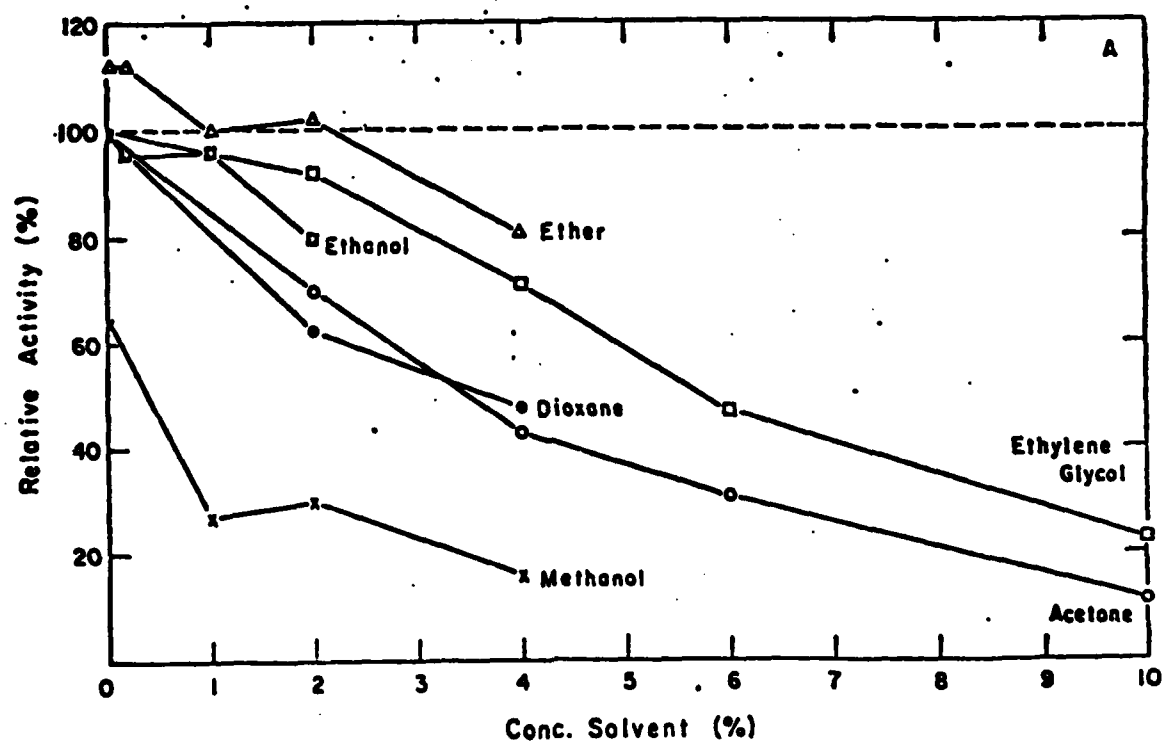


Fig. 10.

Effects of chloramphenicol (Fig. 9) and assorted organic solvents (Fig. 10) on globin biosynthesis.

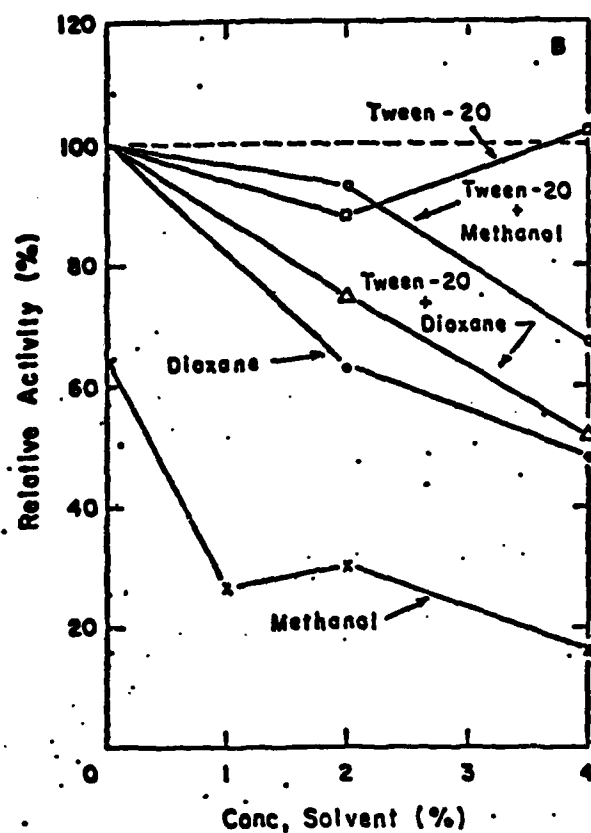


Fig. 11.

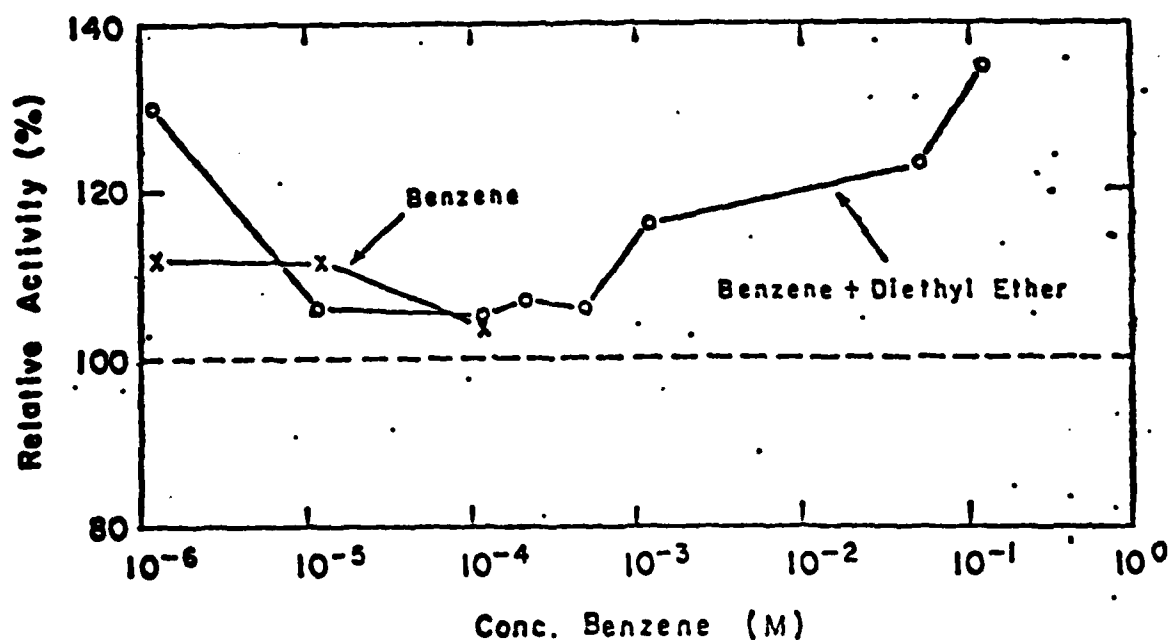


Fig. 12.

Effects of the detergent Tween 20 and various organic solvents on globin formation. (Figs. 11 and 12)

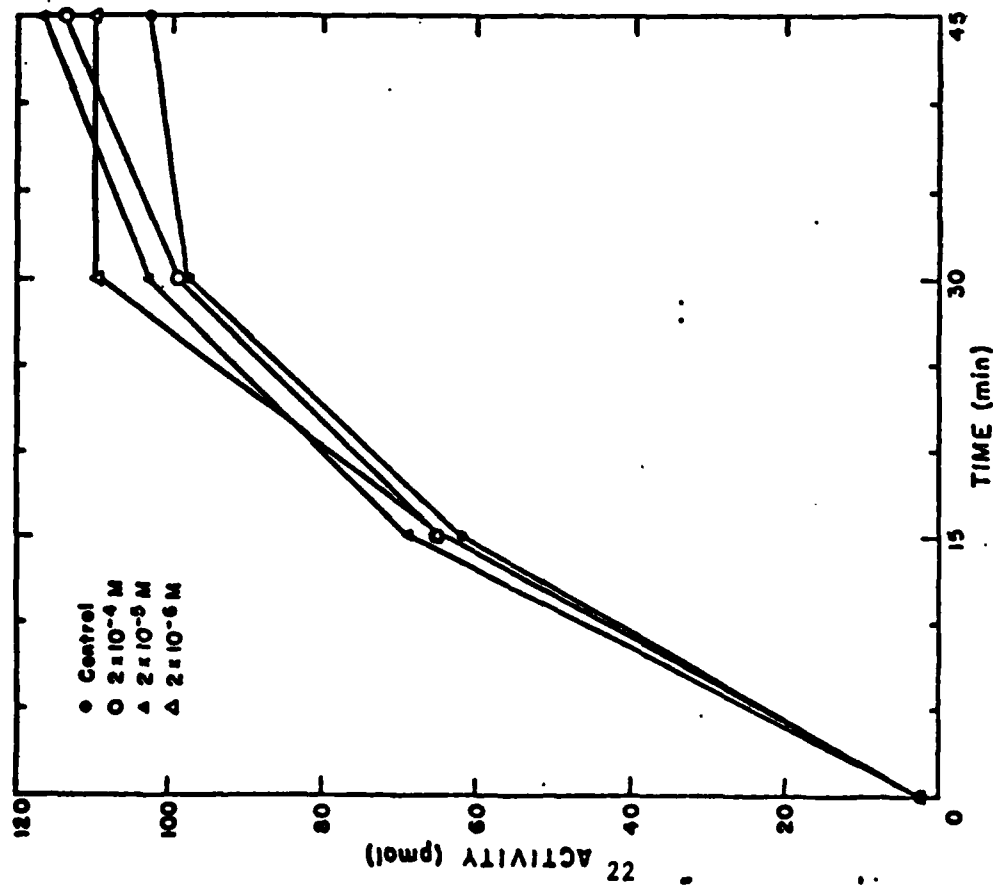


Fig. 13.

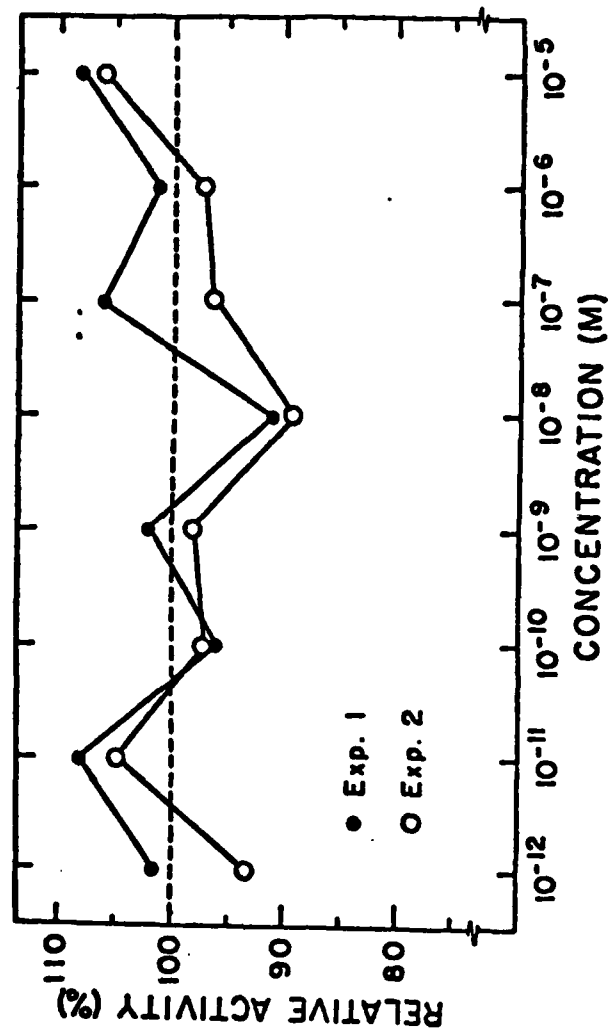


Fig. 14.

Influence of benzene (Fig. 13.) and 1-chloro-2-nitrobenzene (Fig. 14) on globin production.

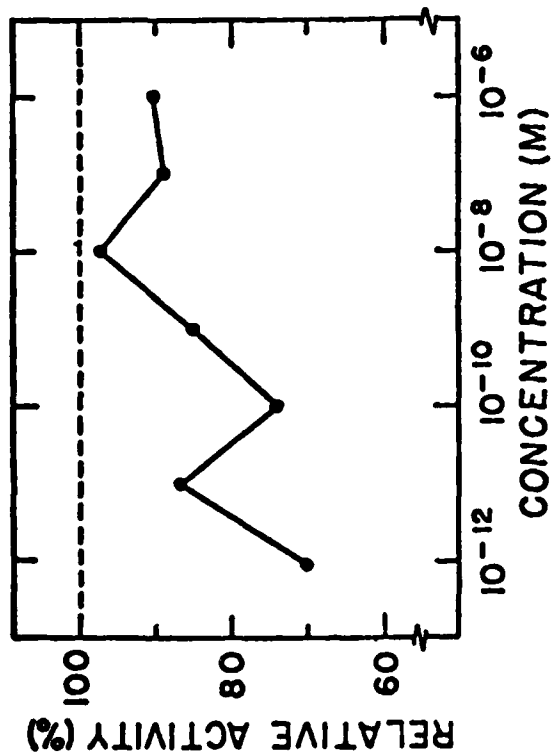


Fig. 15.

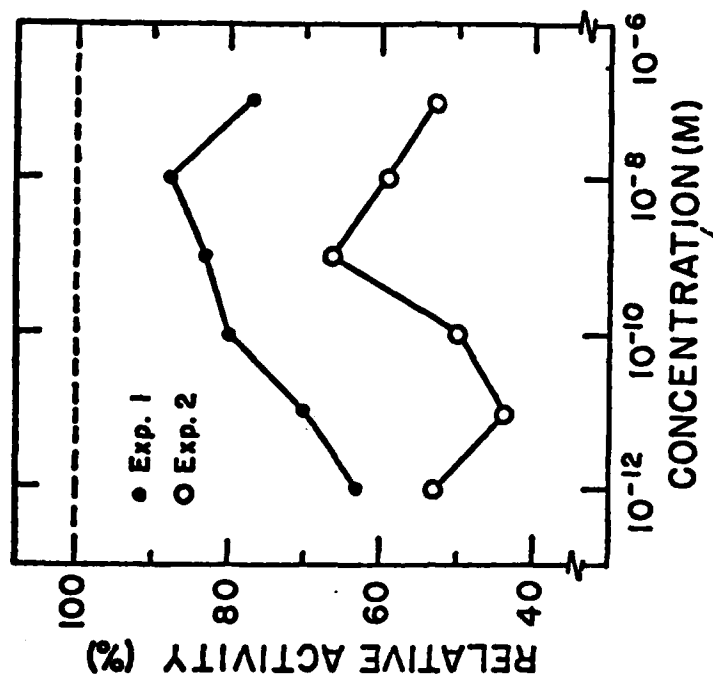


Fig. 16.

Effects of nitrobenzene (Fig. 15) and hexachlorobenzene (Fig. 16) on globin synthesis.

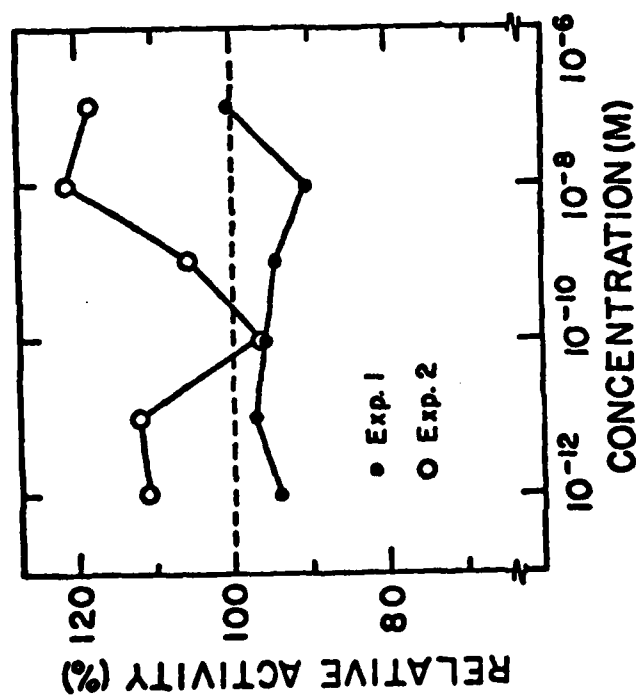


Fig. 17.

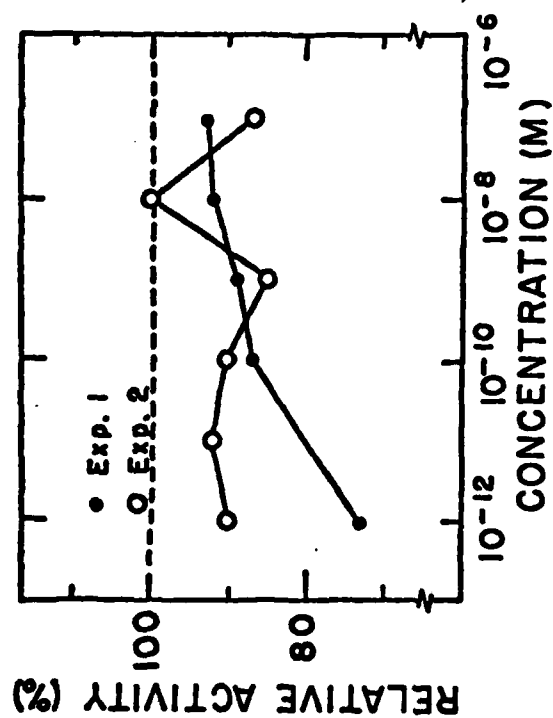


Fig. 18.

Effects of o-chlorotoluene (Fig. 17) and m-chlorotoluene (Fig. 18) on globin biosynthesis.

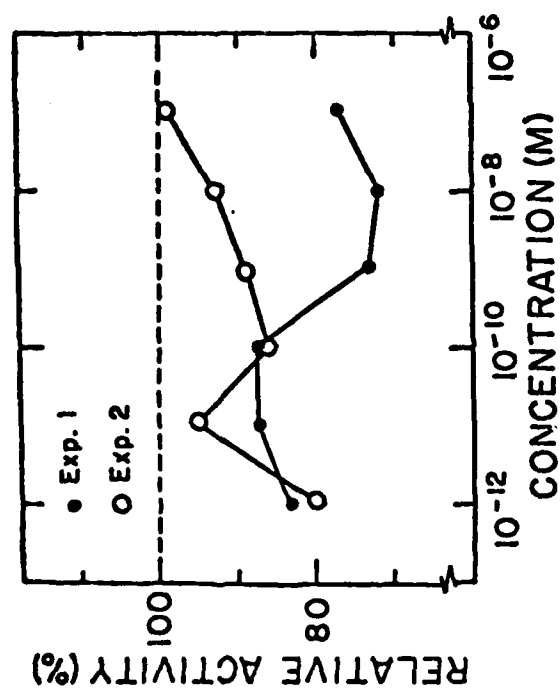


Fig. 19.

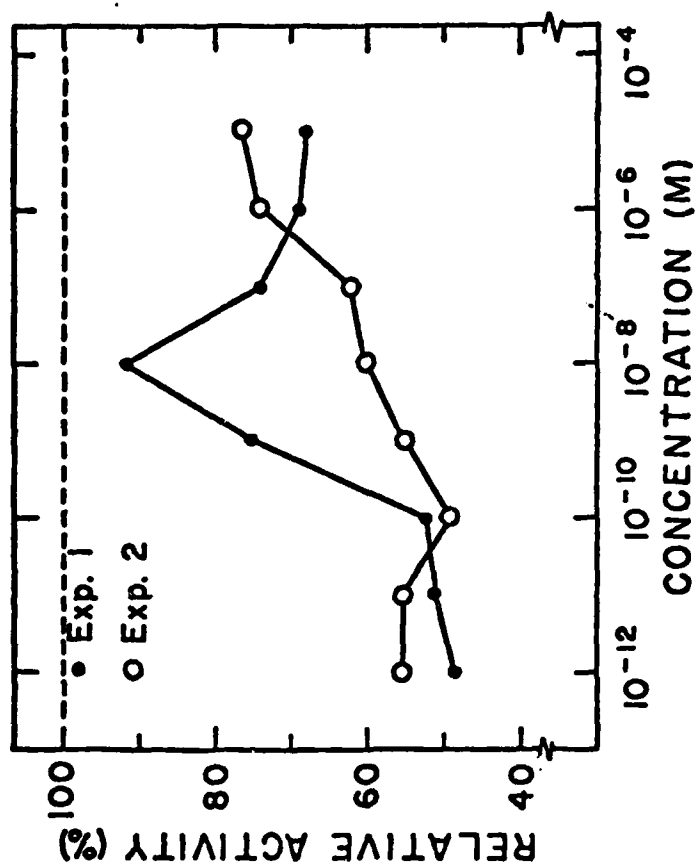


Fig. 20.

Effects on globin formation of p-chlorotoluene (Fig. 19) and 4-nitrotoluene (Fig. 20).

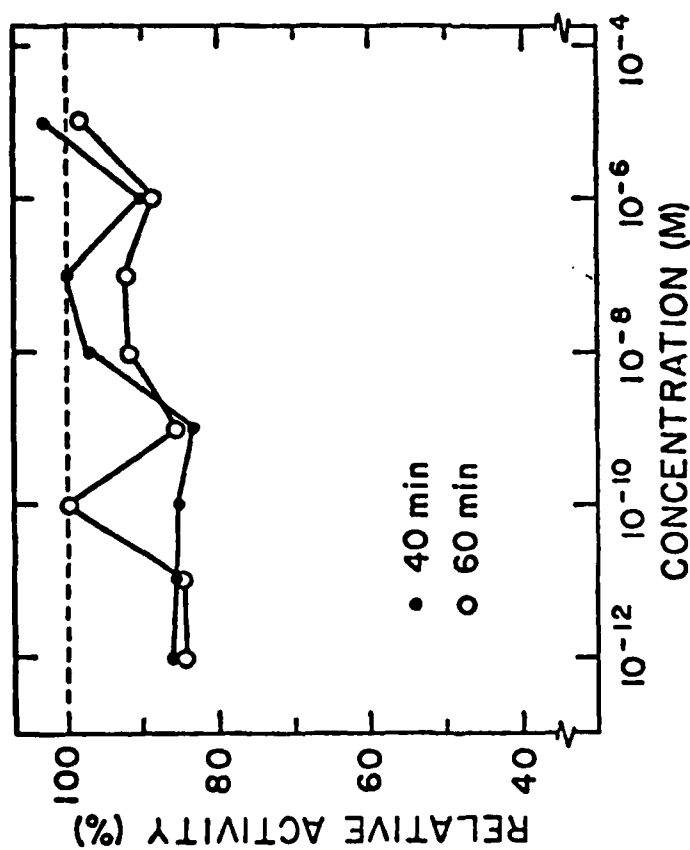


Fig. 21.

Influence of 2-amino-4-nitrotoluene (Fig. 21) and 4-amino-2,6 dinitrotoluene (Fig. 22) on globin formation.

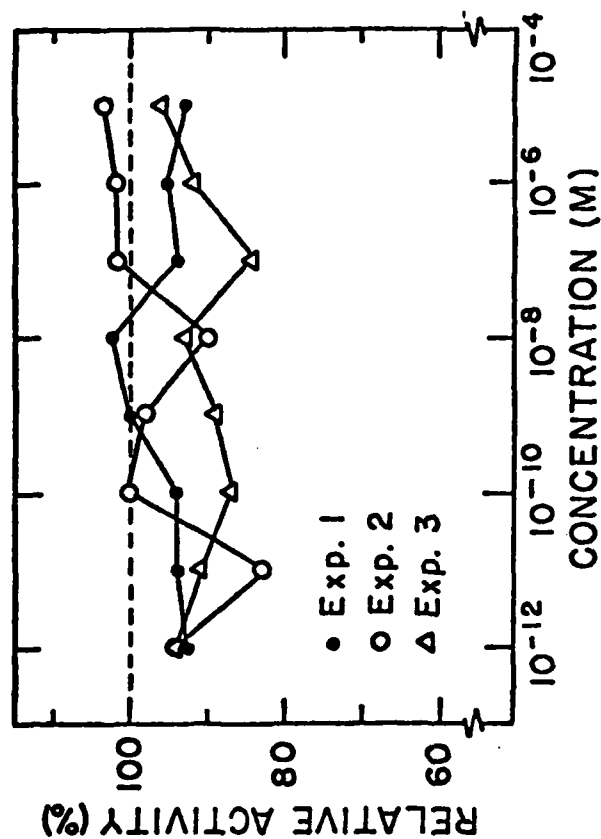


Fig. 22.

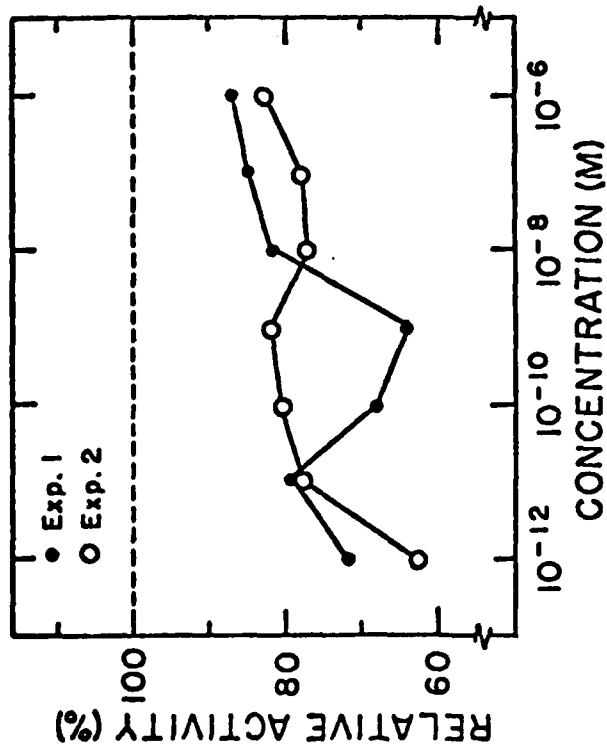


Fig. 23, .

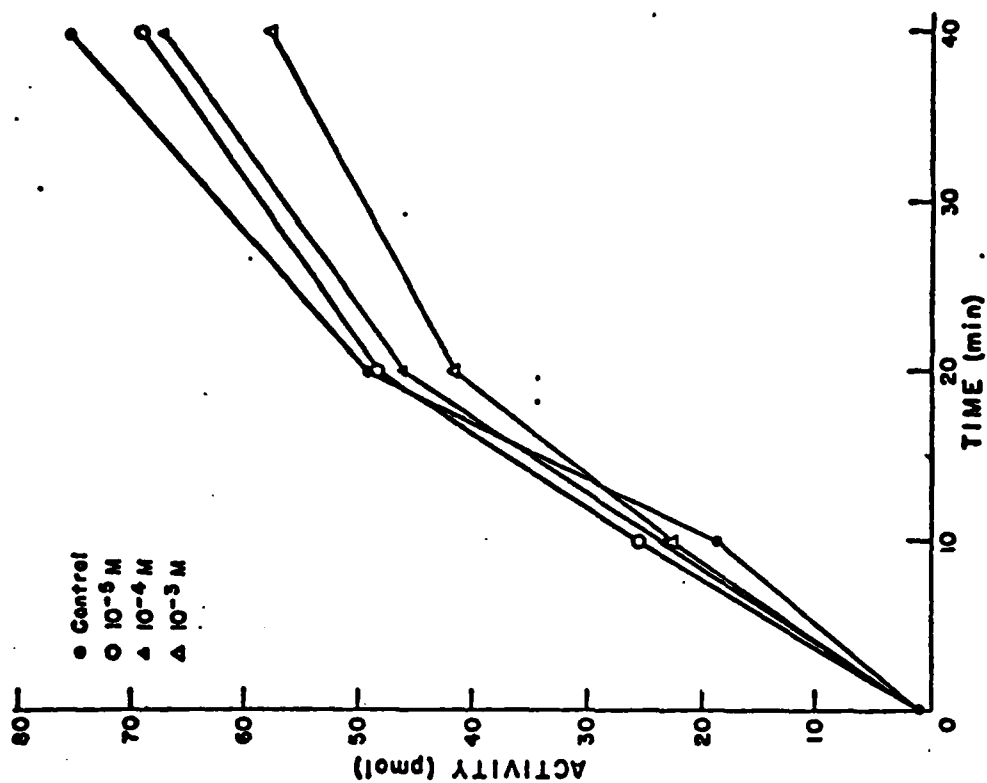


Fig. 24A

Effects on translation of 4-amino-3,5 dinitrotoluene (Fig. 23) and 2,3 dinitrotoluene (Fig. 24).

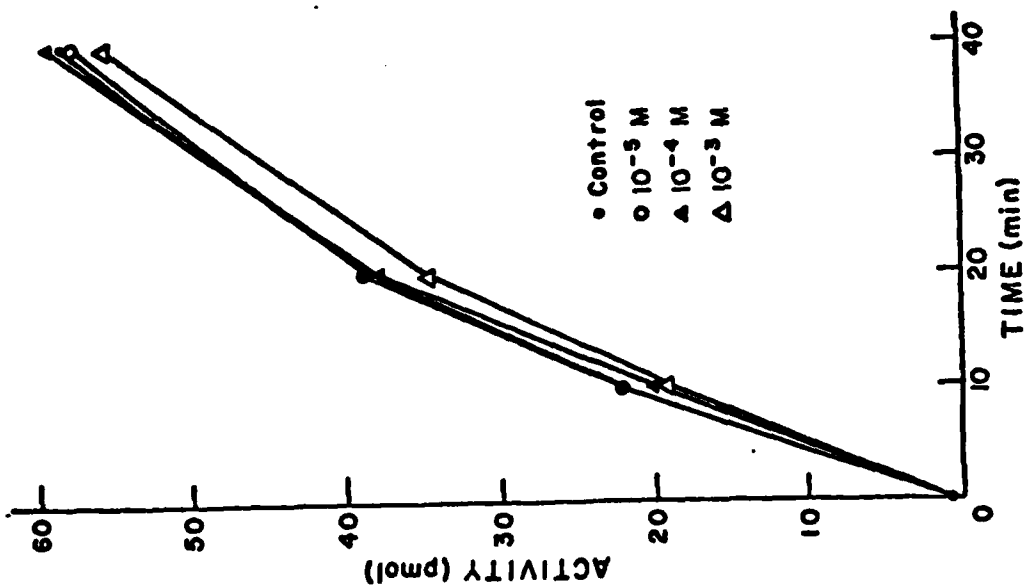


Fig. 24B.

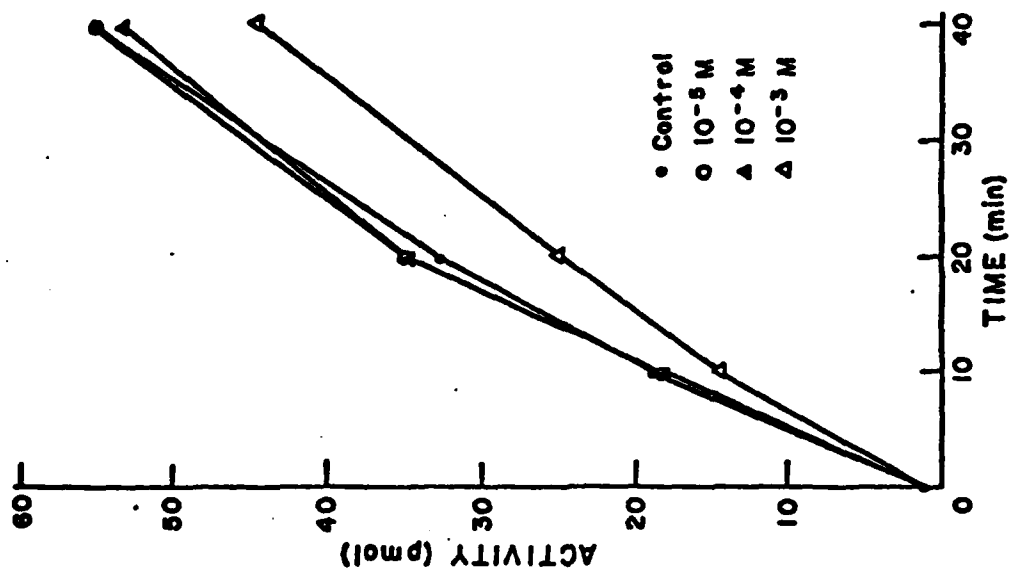


Fig. 24C.

Replicate analyses of effect of 2,3 dinitrotoluene on globin synthesis.

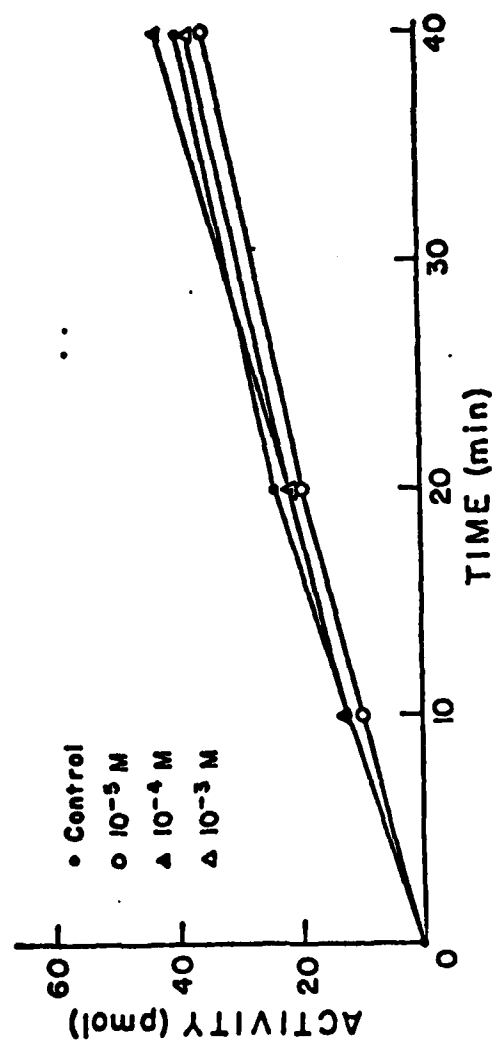


Fig. 25.

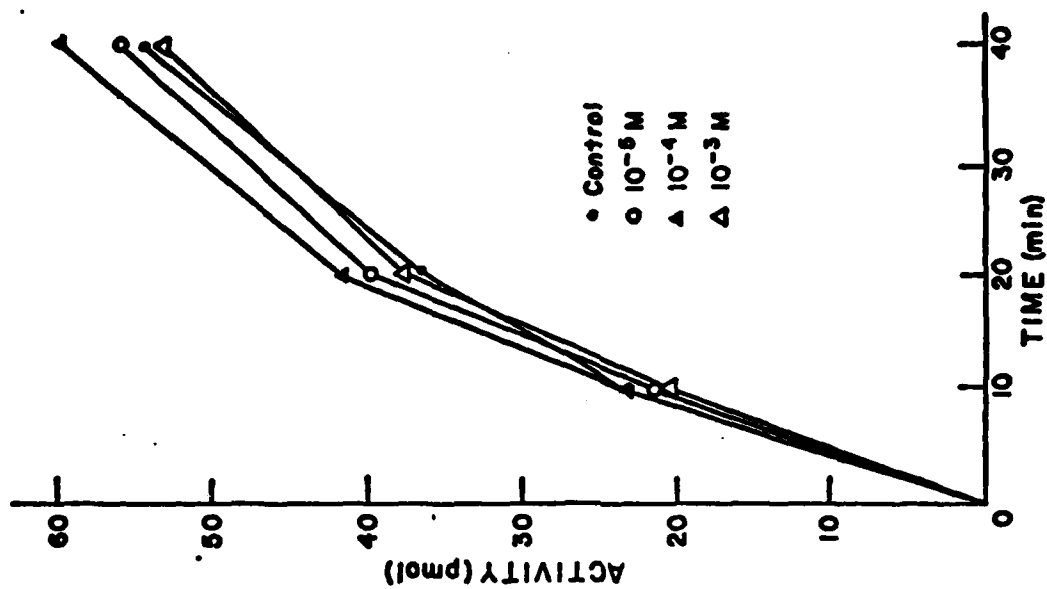


Fig. 26.

Effects on globin biosynthetic activity of 2,4 dinitrotoluene (Fig. 25) and 2,5 dinitrotoluene (Fig. 26).

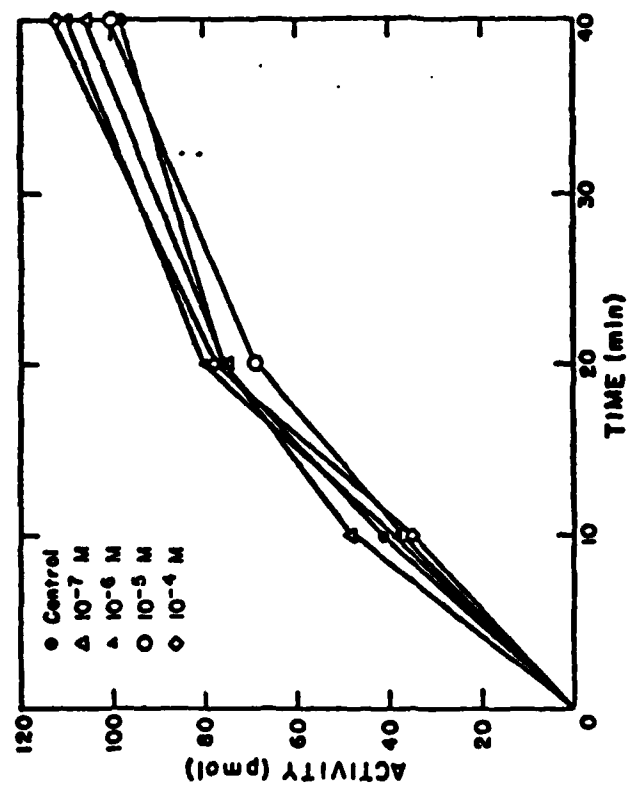


Fig. 27.

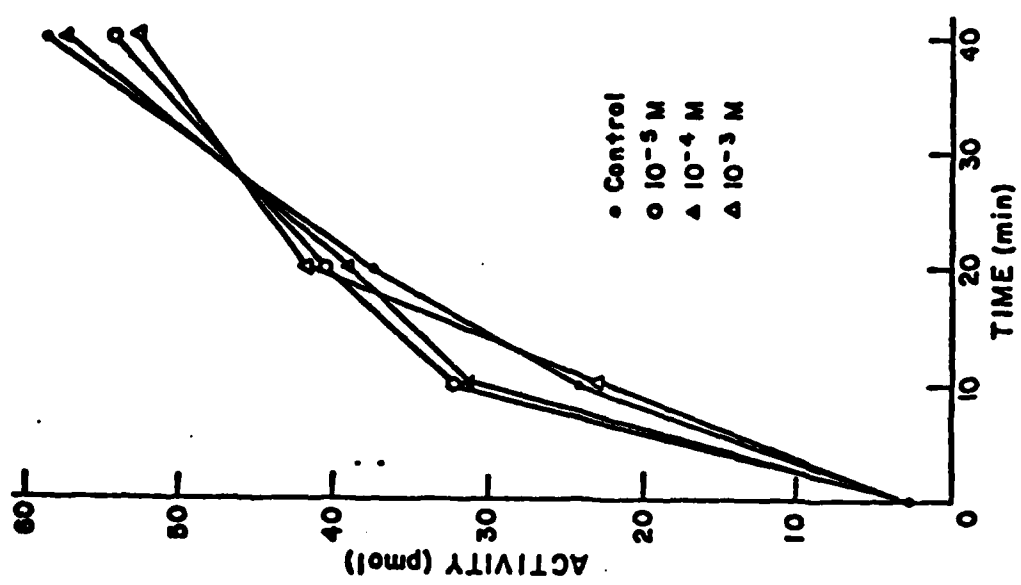


Fig. 28.

Influence of 2,6 dinitrotoluene (Fig. 27) and 3,4 dinitrotoluene (Fig. 28) on globin synthesis.

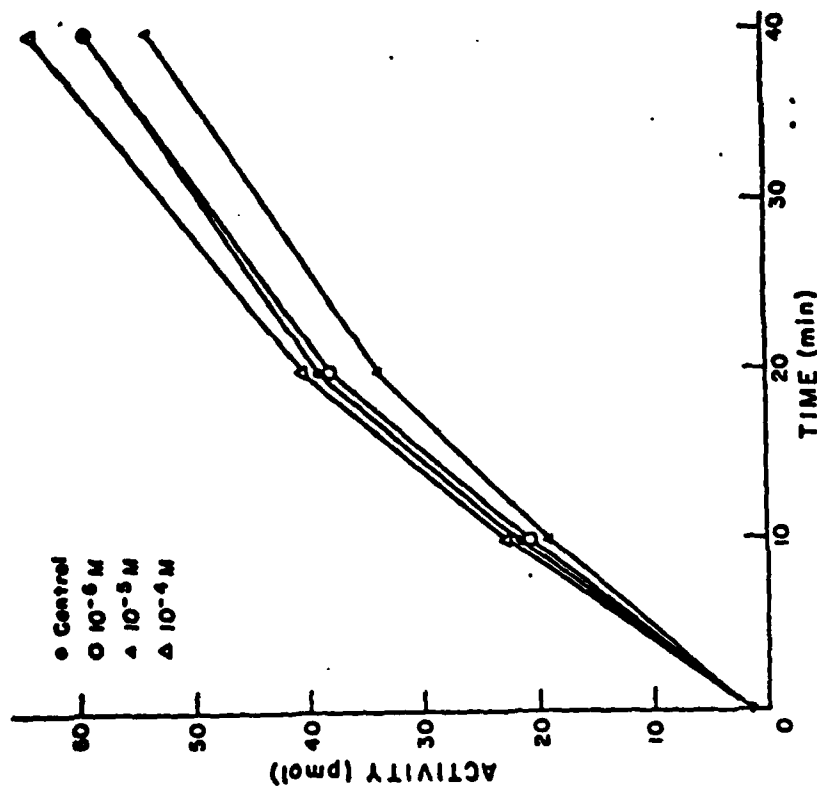


Fig. 29.

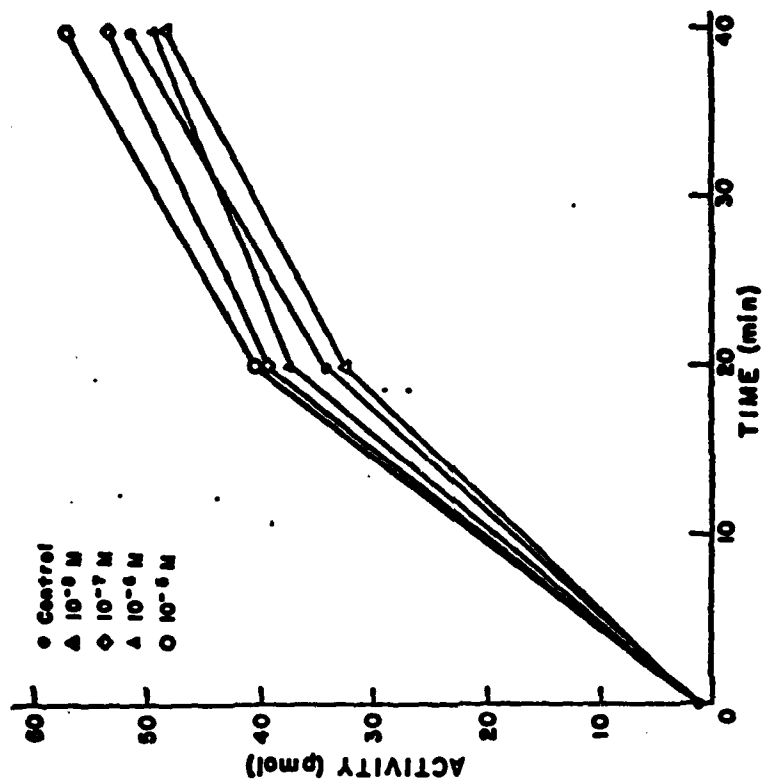


Fig. 30.

Effects of 3,5 dinitrotoluene (Fig. 29) and 2,4,6 trinitrotoluene (Fig. 30) on globin synthesis.

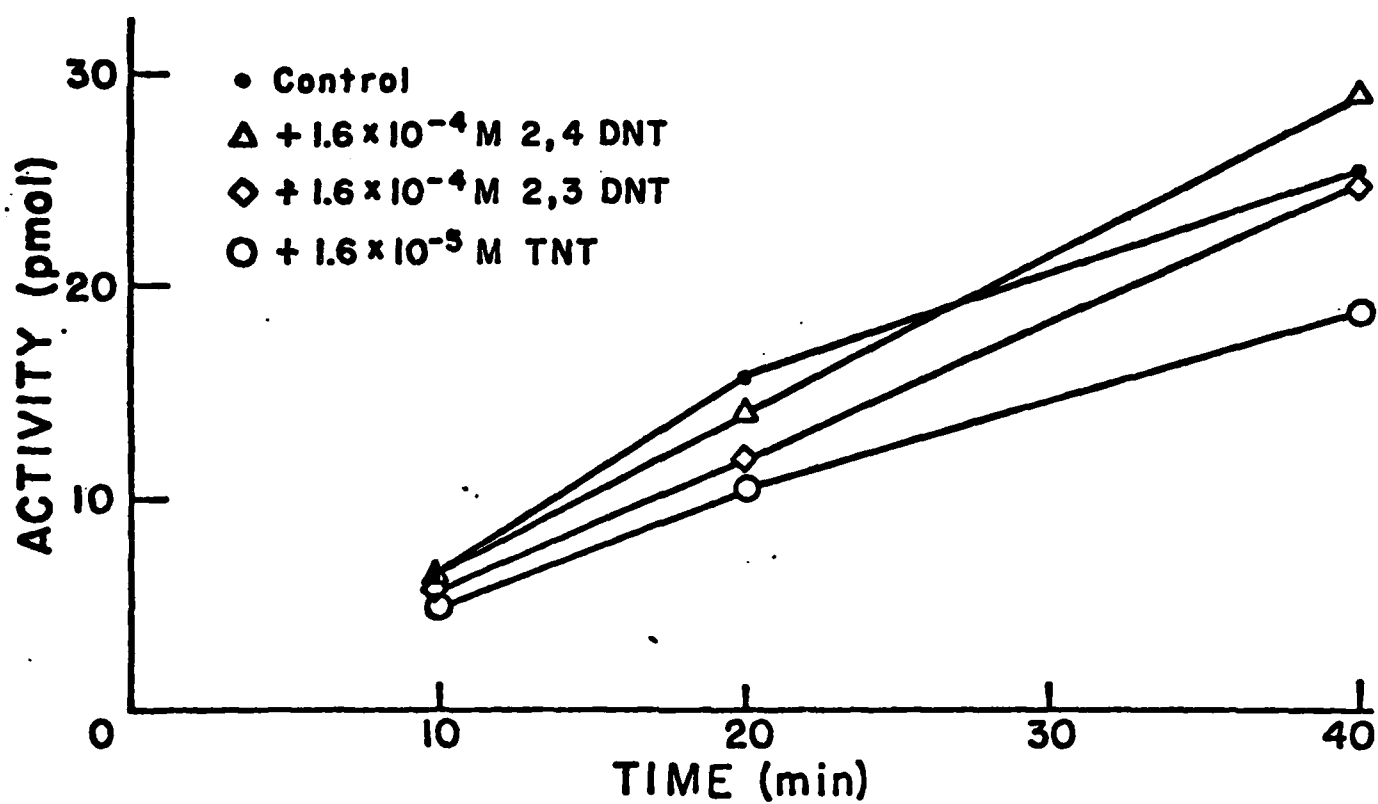


Fig. 31.

Effects of 2,3 dinitrotoluene, 2,4 dinitrotoluene and 2,4,6 trinitrotoluene on globin synthesis in intact reticulocytes.

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