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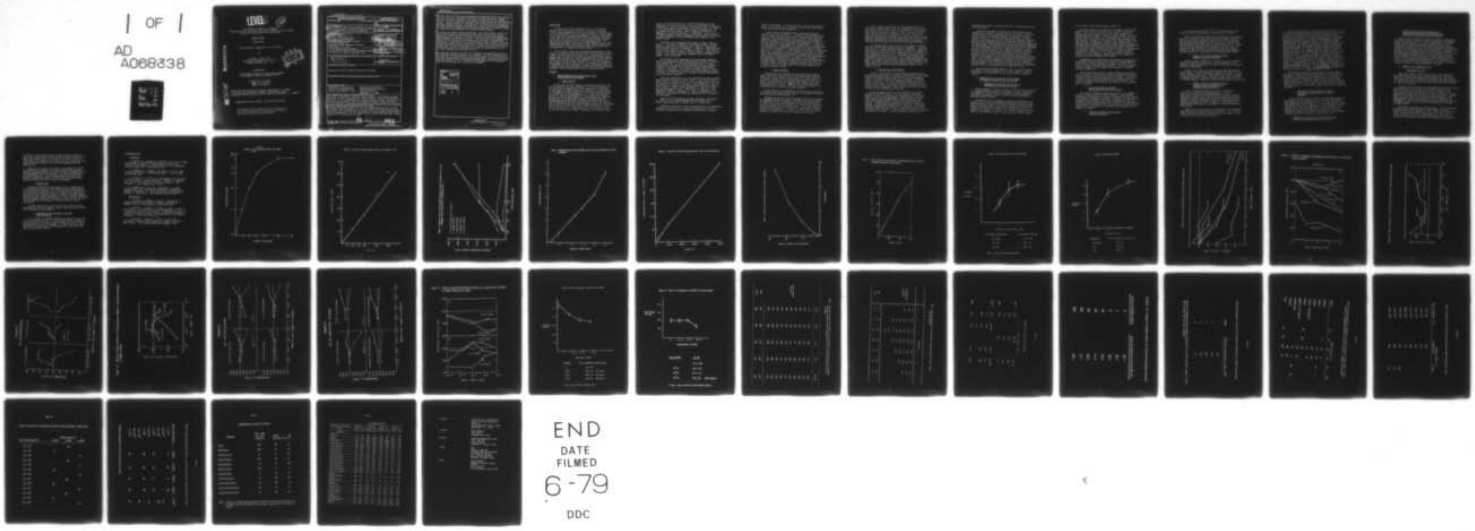

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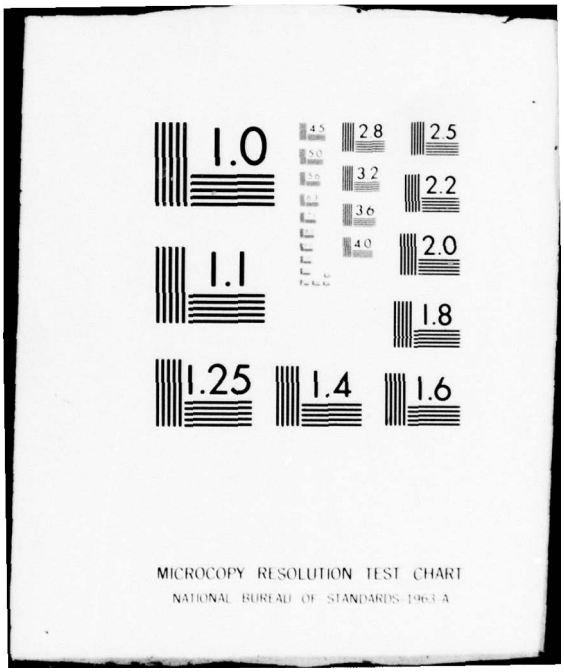
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The Effects of Specific Environmental Pollutants on the Biosynthetic Functions of Mammalian Cells *in vitro*: A Search for Structure/Activity Relationships

ANNUAL REPORT

August 1978

(for the period 1 August 1977 - 31 July 1978)

by

Michael J. Haut, M.D.*
Maurille J. Fournier, Jr., Ph. D. **
Kenneth Goldstein, M.D. ***

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Three test systems-heme synthesis by rat liver homogenates and rabbit reticulocyte lysates, globin synthesis by a cell-free rabbit reticulocyte lysate system and erythroid cell proliferation in a plasma clot culture system have been established and standardized. For the enzyme systems, studies have been done to show the stability, of the enzymes at -70°C.

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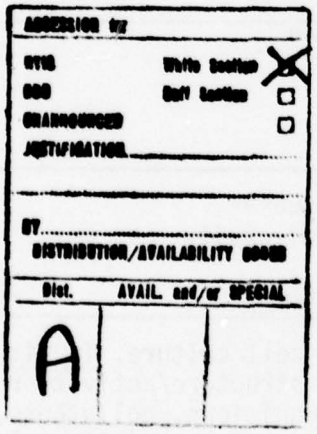
Vehicles used for delivery of the benzene and toluene derivatives were examined for their effects on the globin translation and erythroid culture systems. Based on the results obtained in this study, 1% ethyl alcohol was selected for use as a solvent to introduce the benzene and toluene derivatives into these two systems. Because methanol is routinely used for solubilizing the porphyrin in the heme synthetase reaction, and Tween-20 is used in preparing our homogenates for both the ALA synthetase and heme synthetase reactions, a systematic survey of vehicles was not undertaken for the heme synthesis enzymes.

The effects of benzene, the dinitrobenzenes, the dinitrotoluenes, and the tri-substituted toluenes on heme synthesis and globin synthesis were studied. Benzene was shown to have little deleterious effect on either the enzymes of heme synthesis or on globin translation. Ortho- and para- dinitrobenzene were shown to affect heme and globin synthesis more than meta- dinitrobenzene. Examination of the effects of the dinitrotoluenes on heme synthesis and globin translation revealed that (a) alterations of enzyme activity do not seem to be related solely to the relative positions of the two nitro groups with respect to each other, and (b) in some cases, one enzyme system was sensitive to a particular compound while the other was not.

Dose-toxicity studies of the effect of 23 benzene and toluene derivatives on ALA synthetase and heme synthetase were examined. The most pronounced effects were seen with the dinitrobenzenes, the dinitrotoluenes, the trinitrotoluenes, the amino-dinitrotoluenes, and hexachlorobenzene. Less pronounced effects were seen with benzene, toluene, nitrobenzene, nitrotoluene, the chlorotroluenes, 2-amino-4-nitrotoluene, and 1-chloro-2-nitrobenzene.



Description:

The basic goal of this research project is to determine whether relative in vitro toxicities of a group of compounds arising from breakdown of a particular pollutant can be predicted by (a) examining the relative in vitro toxicities of a small number of such derivative compounds with different structural characteristics, and (b) using structure-activity relationships to predict which others will be most toxic in the in vitro system. The specific test systems we are using are heme synthesis by rat liver homogenates and rabbit reticulocyte lysates, globin synthesis by a cell-free rabbit reticulocyte lysate system, and erythroid cell proliferation in a plasma clot culture system.

The first year's work on this proposal concentrated on three areas: (a) establishing and characterizing the three principal test systems; (b) obtaining toxicity data for a limited number of compounds using all three test system, and examining the relationships between data obtained for a given compound by each system; and (c) utilizing one of the systems, with which it is easier to perform a large number of dose-toxicity studies, to screen a large number of nitrotoluenes and their analogues in order to determine which group of compounds may show significant structure/activity relationships. Progress in each of these three efforts is discussed below.

Progress:

A. Establishment and characterization of the three principal test systems

1. Heme synthesis

In this area, we have focused our attention for the past year on ALA synthetase and heme synthetase in rat liver homogenates. ALA synthetase was measured by a minor modification of the method of Ebert et al (1) Heme synthetase was measured by a modification of the method of Bonkowsky et al (2) In this case the modification was a major one; we used mesoporphyrin IX, rather than protoporphyrin IX. Because mesoporphyrin IX is much easier to solubilize, we were able to use much higher porphyrin concentrations than Bonkowsky et al had been able to achieve with protoporphyrin IX, and we were able to get much higher enzyme activity. Our description of this modification is currently being prepared for publication. Control values for liver ALAS were 1-3 nmol/g protein/0.5 hr and for HS they were 16-27 μ mol/g protein/hr. Each assay was characterized with regard to a number of variables: (a) linearity over

time, (b) relation of activity to protein concentration in the homogenate, (c) reproducibility of the assay performed the same day on multiple aliquots of homogenate from the same liver, (d) stability of the enzyme when frozen as freshly prepared homogenate at -70°C and stored for several weeks, and (e) comparison of activity in homogenates from different animals. In addition, since our assay for heme synthetase involves a substantial modification of previously reported procedures, we have performed kinetic studies of this enzyme as well.

Linearity of ALA synthetase with respect to time is shown in Figure 1. The assay is linear for the first 30 minutes, and then begins to taper off; the decrease in ALA formed vs. time becomes marked beyond 45 minutes of incubation. Linearity of heme synthetase activity with respect to time is shown in Figure 2. This assay is linear for the first 45 minutes, and then appears to become slightly faster. (The slightly lower than expected value at 60 minutes appears to be an artifact).

The relation of ALA synthetase activity and heme synthetase activity to homogenate protein concentration are shown in Figures 3 and 4 respectively. At non-limiting concentrations of alpha-ketoglutarate, the rate of the ALA synthetase reaction increases in a roughly linear manner with increase in protein concentration (Figure 3). Similarly, at non-limiting concentrations of ferrous iron and of mesoporphyrin, the rate of the heme synthetase reaction increases in a linear manner with increase in protein concentration up to 15.36 mg/ml (figure 4).

Within day reproducibility of ALA synthetase and heme synthetase determinations are shown in Tables I and II. The first column of Table I (Day 0) shows results of ten ALA synthetase assays performed the same day on aliquots of pooled rat liver homogenates. Table II shows results of three to eight heme synthetase assays performed on each of seven individual liver homogenates. In the case of both the pooled homogenate (Table I, Day 0) and the individual liver homogenates (Table II, Homogenate 1; Table II, Homogenate 2; etc), there was a high degree of within day reproducibility of assays on the same sample.

Stability of ALA synthetase and heme synthetase, when frozen at -70°C as freshly prepared homogenates and stored for up to several weeks, are shown in Tables I and III, respectively.

Significant variations in heme synthetase activity in homogenates prepared from different rat livers are demonstrated in Table II. Preliminary studies with ALA synthetase indicates that the same is

true for that enzyme. It can be seen that variation from animal to animal is significant, and is certainly greater than that from day to day with frozen pooled homogenates.

Kinetic studies performed on heme synthetase are shown in Figures 5 and 6 and in Table IV. In Figure 5, the concentration of iron is varied, with the concentration of mesoporphyrin remaining constant at 1000 nanomoles per reaction aliquot. Within the concentration range of iron tested, the enzyme activity increased in a nearly linear fashion with increased iron concentration. (There is a slight tapering at the highest iron concentration). In Figure 6, the concentration of mesoporphyrin is varied, with the concentration of iron remaining constant at 500 nanomoles per reaction aliquot. When activity (ordinate) is plotted against mesoporphyrin concentration (abscissa), the resulting curve is hyperbolic, but does not plateau even at 2650 nanomoles of mesoporphyrin, suggesting that the saturation level for mesoporphyrin may be even higher than 2650 nanomoles per aliquot of reaction mixture. In Table IV, a preliminary experiment showing the effect of added heme on heme synthetase activity is demonstrated. Although the results are not entirely clear-cut from this initial experiment, the general pattern seems to be one of enzyme inhibition at heme levels of 20 or more nanomoles per aliquot of reaction mixture.

2. Globin synthesis

Reticulocytes were obtained from New Zealand white rabbits after induction of reticulocytosis by phenylhydrazine injection, essentially as described by Crystal *et al.* (3). 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. Membrane-free lysates prepared by this method can be stored at -85°C for at least one year with no significant loss in ability to synthetize globin.

Globin synthesis was determined by measuring the incorporation of ^{3}H-leucine into acid-insoluble protein. The procedures used were those described by Woodard *et al.* (4).

Although one need only add a radiolabeled amino acid to monitor the endogenous protein biosynthetic activity, the rate and extent of translation can be increased significantly by the addition of: (1) hemin, which blocks the formation of a translational inhibitor, and (2) an energy (ATP) generating system - in our case creatine kinase/creatine phsophate. The effects of adding other components required for translation i.e., amino acids, ATP, GTP, etc., were tested but no additional increase in activity was observed.

Figure 7 shows a time course for the incorporation of ^{3}H-leucine into globin protein using the supplemented lysate system from rabbit reticulocytes. Under the conditions of our assay the rate of amino acid incorporation is linear for about 40 minutes after which time there is a decrease in activity. While it is clear that the activity continues for at least an hour we have not measured incorporational activity beyond that time. Similar results have been obtained with four other preparations, although the relative activities vary from 50-120% of that shown (see Table V).

In a typical control assay, from 2-15 pico moles of leucine have been incorporated after 60 minutes of incubation. This level of activity is in good agreement with that reported by a number of other workers in the field. With regard to precision, analysis of quadruplicate data points from 21 independent assays performed with five independent preparations of lysate show the variance due to experimental error to be less than $\pm$ 7-8%. When evaluating the effects of solvents and pollutants, incorporation measurements were made in duplicate at both 40 and 60 minutes; control reactions (no agent) were always carried out in parallel and the activity of the lysate containing the test substance compared directly to the control activity.

3. Erythroid cell proliferation

A CFU-E culture system was established and standardized for dog bone marrow aspirates. In essence, nucleated cells are isolated from the aspirated marrow by centrifugation in Ficoll-hypaque, placed in a defined culture medium, and incubated in microtiter wells for 48 hours at 39°C in a humidified incubator with a 4% carbon dioxide level. Following incubation, the plasma clots are placed on glass slides and stained. Erythroid colonies are then counted using the microscope; these colonies are defined as groups of eight or more hemoglobin staining cells.

To date the major activity in the laboratory has been to standardize this CFU-E assay. The first several months were devoted to obtaining appropriate culture material in sufficient quantity to use for the experiments. Fifteen batches of bovine serum albumin were screened. One was found to have excellent activity in promoting CFU-E in dogs and has been purchased in quantity. Twenty-three different lots of fetal calf serum were screened. Two were found appropriate for the assay. Only one of these was commercially available in a reasonable amount and was obtained by our laboratory. These have been the two major variables in setting up the assay. The other necessary supplies, such as

erythropoietin, bovine citrated plasma, etc., are obtained and have been working well.

As soon as the supply of appropriate culture materials was completed, work began on standardizing the CFU-E assay. Variation in efficiency of culture techniques was a significant problem initially, and still presents problems occasionally. The major reasons for this are: (1) The cell separation technique of dog bone marrow in which nononuclear cells are separated from whole bone marrow by Ficol-hypaque sedimentation has not yielded pure mononuclear cell populations. It has been both our experience and the experience of other investigators that this is uniformly difficult to accomplish due to size and shape similarities between mononuclear and non-mononuclear marrow precursor cells. We have, therefore, attempted to simply use the Ficol-hypaques sedimentation to eliminate all mature red cells obtained from bone marrow aspiration and not to attempt at the present time to further purify bone marrow precursor cells. (2) The amount of nutrients available to the marrow precursor cells varies. We are attempting to diminish our cell inoculum for culture to better allow sufficient nutrients for cell maturation and development, and are proceeding with the addition of supplemental nutrients to our culture material to increase efficiency.

The relationship between number of cells in initial inoculum and number of colonies formed per 10^5 cells is shown in Figure 8. Erythropoietin dose response for the culture system is shown in Figure 9.

B. Comparative toxicity data on limited number of compounds using all three test systems

1. Examination of vehicles for delivery of the benzene and toluene derivatives

Because methanol is routinely used for solubilizing the porphyrin in the heme synthetase reaction, and Tween-20 is used in preparing our homogenates for both the ALA synthetase and heme synthetase reactions, a systematic survey of vehicles was not undertaken for the heme synthesis enzymes.

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 VII.

Not surprisingly, the solvents (and detergent) tested varied greatly in their effects on translation. In order of deceasing potency, the relative toxicities of the simple solvents at 2% concentration were: methanol, acetone, dioxane, ethanol, Tween-20, ethylene glycol, and ether. At 4% concentration of solvent, the relative toxicities were similar except that ether was more toxic than Tween-20. (Ethanol was not done at this concentration). Dose response studies on methanol, acetone, ethanol, ethylene glycol, and ether show increased inhibition at higher solvent concentrations. In three of the cases (methanol, ethanol, and ether), there was little inhibition until the solvent reached a specific concentration. The detergent, Tween-20, had essentially no effect on protein synthesis over the range of 0-2%, and only moderate inhibitory effect at 4%; the presence of Tween-20 neutralized somewhat the inhibitory effects of methanol and dioxane.

A comparable, but considerably less extensive survey was done of vehicles in the erythroid culture system. It became apparent that control cultures incubated with ethanol did better than our control cultures incubated without ethanol. This did not appear to be due to changes in pH but seemed to be related to increased nutrient availability.

Based on the above results, 1% ethyl alcohol was selected for use as a solvent to introduce the benzene and toluene derivatives into the translation and erythroid culture systems. In an effort to make the three systems as comparable as possible, the effect of 1% ethyl alcohol on ALA synthetase and heme synthetase will be tested, and ethanol will be used as the vehicle in this system as well, if feasible.

2. Purity of benzene and toluene derivatives used in toxicity studies

The aromatic compounds studied came primarily from commercial sources, principally Aldrich Chemical Company. Some compounds unavailable from commercial sources were obtained from the Division of Experimental Therapeutics at WRAIR. We tested for impurities by subjecting each compound to thin-layer chromatography using two different solvent systems benzene-ethyl acetate 1:4, and acetonitrile. Each compound tested showed only a single spot on migration on pre-coated silica gel plates (E.M. Laboratories, 500 Executive Blvd., Elmsford, NY 10523).

3. Effect of benzene on heme synthesis and globin synthesis

In the concentration range 10^{-6} to 10^{-3}, benzene had little effect on either ALA synthetase or heme synthetase (Table XI).

Figure 12 and Table VII show the dose-response results obtained thus far for benzene in the globin translation system. Because of the low solubility of benzene in water (8.9×10^{-3} M) assays were performed for benzene alone over the concentration range 10^{-6}-10^{-3} M and benzene in 4% diethyl ether over the range 10^{-6}-10^{-1} M. (Ether at the 4% level inhibits activity by about 20% -Figure 10.) The results show that benzene has no deleterious effect on protein synthesis in the cell-free system used here and indeed, appears to stimulate amino acid incorporation at all concentrations tested.

4. Effect of the dinitrobenzenes on heme synthesis and globin synthesis

Figure 13 shows a comparison of the activities of the dinitrobenzenes on ALAS and HS activities. One may note that in concentration ranges greater than 10^{-5} mol/l, as HS activity increases ALAS activity decreases.

Figure 14 and Table VIII show the results obtained when the three isomers of dinitrobenzene were tested on the rabbit cell-free globin synthesis system. All three compounds are soluble in water at the concentrations tested. Inspection of the results show that 1,3-DNB has essentially no effect over the range tested (6×10^{-8} - 6×10^{-4} M), whereas 1,4-DNB and 1,2-DNB inhibit at most concentrations.

5. Effect of the dinitrotoluenes on heme synthesis, globin synthesis, and erythroid cell proliferation

Figure 15 gives the comparison of the dinitrotoluenes' effect on ALA synthetase and heme synthetase. At concentrations similar to the dinitrobenzenes, the effect is not as great, which suggests that there may be some interaction between the methyl and nitro groups that offset the enhancing-inhibiting effects. A similar trend may be noted with trinitrotoluenes and aminodinitrotoluenes as shown in Figure 16. The addition of an amino group to the aromatic ring appears to lessen the decrease in ALAS activity.

When five of the six possible dinitrotoluene (DNT) homologues were tested (only 3,5-DNT has yet to be tested) in the cell-free globin translation system, the dose-response data shown in Figure 17A and 17B and Table IX were obtained.

Testing was performed over the concentration range of 10^{-12} to 10^{-4}. All of the DNT's tested inhibited translation to some extent although to varying degrees and at different concentrations. Variation of inhibition with concentration of DNT did not seem to be a simple relationship. For many of the compounds, inhibition appeared to be more pronounced at their highest and/or lowest concentrations. For example, inhibition of protein synthesis by 2,3-DNT and 2,5-DNT were more pronounced at lower concentrations than at higher ones. For 2,3-DNT, relative activity of globin translation was 80% for 10^{-12}M vs 109% for 10^{-8}M, 94% for 10^{-6}M, and 91% for 10^{-4}M. For 2,5-DNT, relative activity of globin translation was 70% for 10^{-12}M and 68% for 10^{-8}M, vs 86% for 10^{6}M and 92% for 10^{-4}M. For other compounds, inhibition of protein synthesis was more pronounced at higher concentrations than at lower ones. For example, relative activity of globin translation was 70% for 10^{-4}M 2,6-DNT and 67% for 10^{-6}M, vs 83% and 94% for 10^{-8} and 10^{-12}M concentrations of this compound. From the data in Table IX, it appears that the order of inhibtory effectiveness of the dinitrotoluenes tested is: (1) most inhibitory: 2,6-DNT (high doses) and 2,5-DNT (low doses); (2) intermediately inhibitory: 3,4-DNT and 2,3-DNT; and (3) least inhibitory: 2,4-DNT.

Preliminary observations of the effects of graded concentrations of 2,6-DNT and of 3,4-DNT on erythroid colony formation are shown in Figures 18 and 19, respectively. For 2,6-DNT, inhibtion of colony formation was present at each concentration tested (figure 18). Inhibition increased with increasing dose of the 2,6-DNT (21% inhibition at 10^{-6}M; 34% inhibition at 10^{-5}M; 38% inhibition at 10^{-4}M). For 3,4-DNT, inhibition of colony formation was not present at 10^{-7}M or 10^{-6}M; however, there was 33% inhibition at 10^{-5}M.

6. Correlation of data obtained using two or three of the test systems for specific compounds

Little correlation can be done at this time among systems for two reasons: (a) The erythroid culture system has only recently been standardized and characterized, and only two compounds have been examined with this systems so far. (b) Results of the studies of effects of the various compounds on protein synthesis are not entirely clear. The fact that both high and low levels of the benzene and toluene derivatives inhibit translation, whereas intermediate levels do not, must be shown to be a real phenomenon and not an artifact produced by a non-optimized system.

C. Utilization of dose-toxicity studies of a large number of nitrotoluenes and their analogues

on ALA synthetase and heme synthetase to determine which groups of compounds may show significant structure-activity relationships

Table XI shows preliminary screening studies of 23 benzene and toluene derivatives. Benzene, toluene, nitrobenzene, nitrotoluene and the chlorotoluenes (o-, m-, and p-) demonstrate only minimal effects on ALA synthetase and heme synthetase 2-amino-4-nitrotoluene and 1-chloro-2-nitrobenzene show slightly more pronounced alterations in enzyme activity. The dinitrotoluenes, trinitrotoluenes, and amino-dinitrotoluenes produce for the most part, pronounced decreases in ALA synthetase activities and increases in HS activities, with the degree of enzyme alteration varying with the particular structure-Hexachlorogenzene appears to increase heme synthetase activity without significantly decfeasing ALA synthetase activity; in fact, it increases ALA synthetase activity at a concentration of 10^{-3}M. Aniline's effect on ALA synthetase is minimal; it produces a moderate increase in heme synthetase at high doses.

D. Summary of work completed so far

1. Experimental results

Three test systems - heme synthesis by rat liver homogenates and rabbit reticulocyte lysates, globin synthesis by a cell-free rabbit reticulocyte lysate system, and erythroid cell proliferation in a plasma clot culture system have been established and standardized. For the enzyme systems, studies have been done to show the stability, at -70°C, of the enzymes.

Vichicles used for delivery of the benzene and toluene derivatives were examined for their effects on the globin translation and erythroid culture system based on the results attached in this study 1% ethyl alcohol was selected for use as a solvent to introduce the benzene and toluene derivatives into these two systems. Because methanol is routinely used for solubilizing the prophyrin in the heme synthetase reaction, and Tween-20 is used in preparing our homogenates for both the ALA synthetase and heme synthetase reactions, a systematic survey of vihicles was not understaken for the heme synthesis enzymes.

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the effects of the dinitrotoluenes on heme synthesis and globin translation revealed that (a) alterations of enzyme activity do not seem to be related solely to the relative positions of the two nitro groups with respect to each other, and (b) in some cases, one enzyme system was sensitive to a particular compound while the other was not.

Dose-toxicity studies of the effect of 23 benzene and toluene derivatives on ALA synthetase and heme synthetase were examined. The most pronounced effects were seen with the dinitrobenzenes, the dinitrotoluenes, the trinitrotoluenes, the amino-dinitrotoluenes, and hexachlorobenzene. Less pronounced effects were seen with benzene, toluene, nitrobenzene, nitrotoluene, the chlorotoluenes, 2-amino-4-nitrotoluene, and 1-chloro-2-nitrobenzene.

2. Problem areas

The major problem area at the present time is standardization of the three test systems so that results for each system are not only qualitatively consistent, but also quantitatively reproducible. In standardizing the system, particular attention will have to be paid to the globin synthesis studies, in order to determine whether inhibition by the lowest concentration (10^{-12}M) of toluene derivatives and the frequent occurrence of inhibition at both high and low (but not intermediate) concentrations of the derivatives is real or artifactual.

A second problem area is that the toxicity studies have only been done once for each compound. Several repeat studies will have to be performed for each compound.

3. Usefulness of this approach to in vitro toxicity testing

It is too early to determine whether the approach we are using will be of practical use for in vitro toxicity testing. Hopefully, we will be able to show that the three systems give similar data on relative toxicity of a given compound. If that is the case, then the simplest of the assays will have predictive value, and can be used as a preliminary screen.

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1. Williams, H., Johnson, D., Haut, M., and Altstatt, L. Structure-activity relationships of aromatic compounds on enzymes involved in heme synthesis. Blood 50:88, 1977

2. Williams, H., Johnson, D., Slater, S., Page, J., Haut, M., and Altstatt, L. A technique for assessing hematologic toxicity of benzene and some of its derivatives. (Presented at the May 1978 meeting of the American Society for Pharmacology and Experimental Therapeutics.) Fed Proc 37:643, 1978

3. Williams, H., Johnson, D., Haut, M. and Altstatt, L. The effect of certain aromatic compounds on enzymes involved in heme synthesis. Army Science Conference Proceedings. 1978

Figure 1.
Linearity of ALA synthetase reaction with respect to time

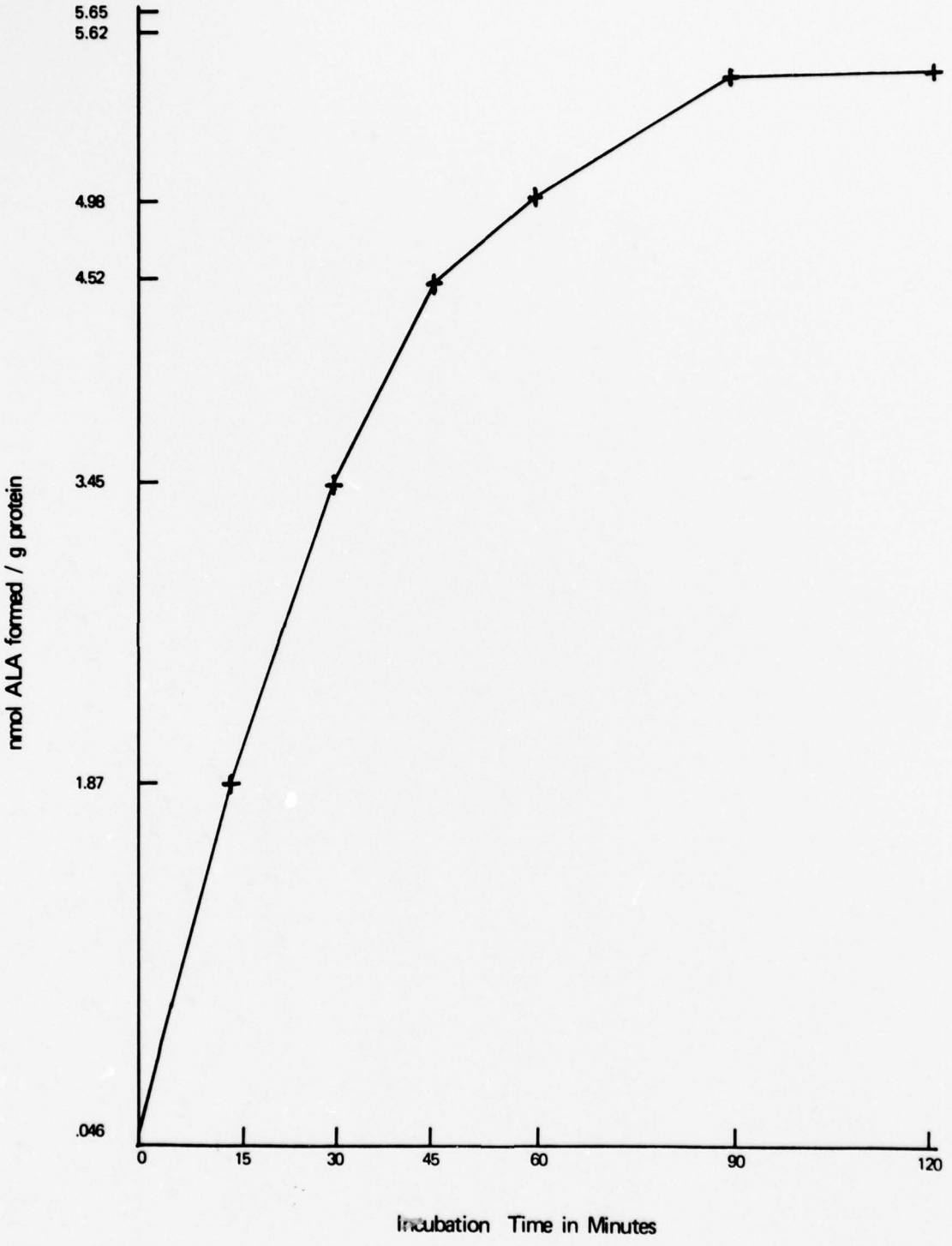


Figure 2. Linearity of Heme Synthetase reaction with respect to time

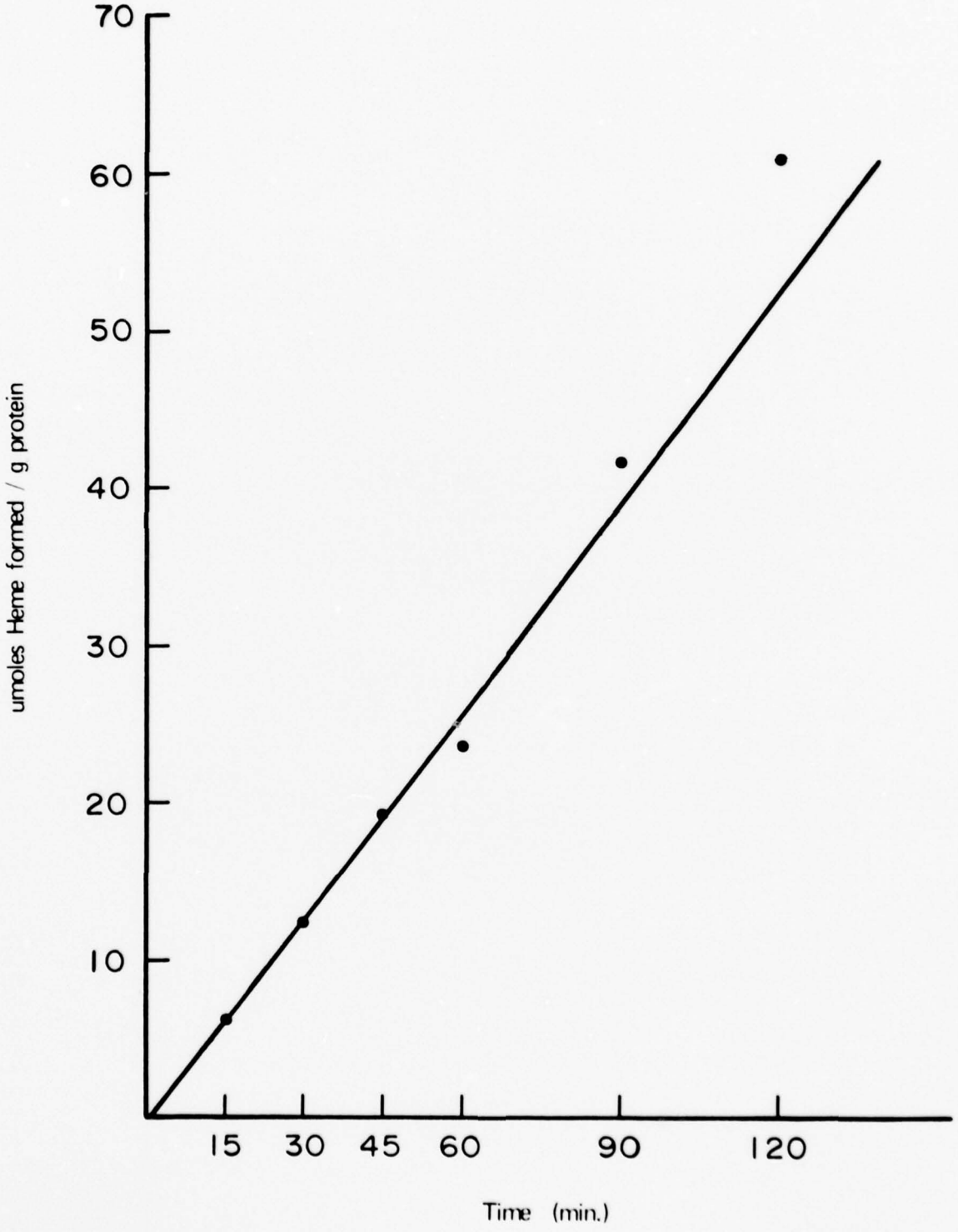


Figure 3. Relation of rate of ALA Synthetase reaction to protein concentration of rat liver homogenate and to concentration of alphaketoglutarate.

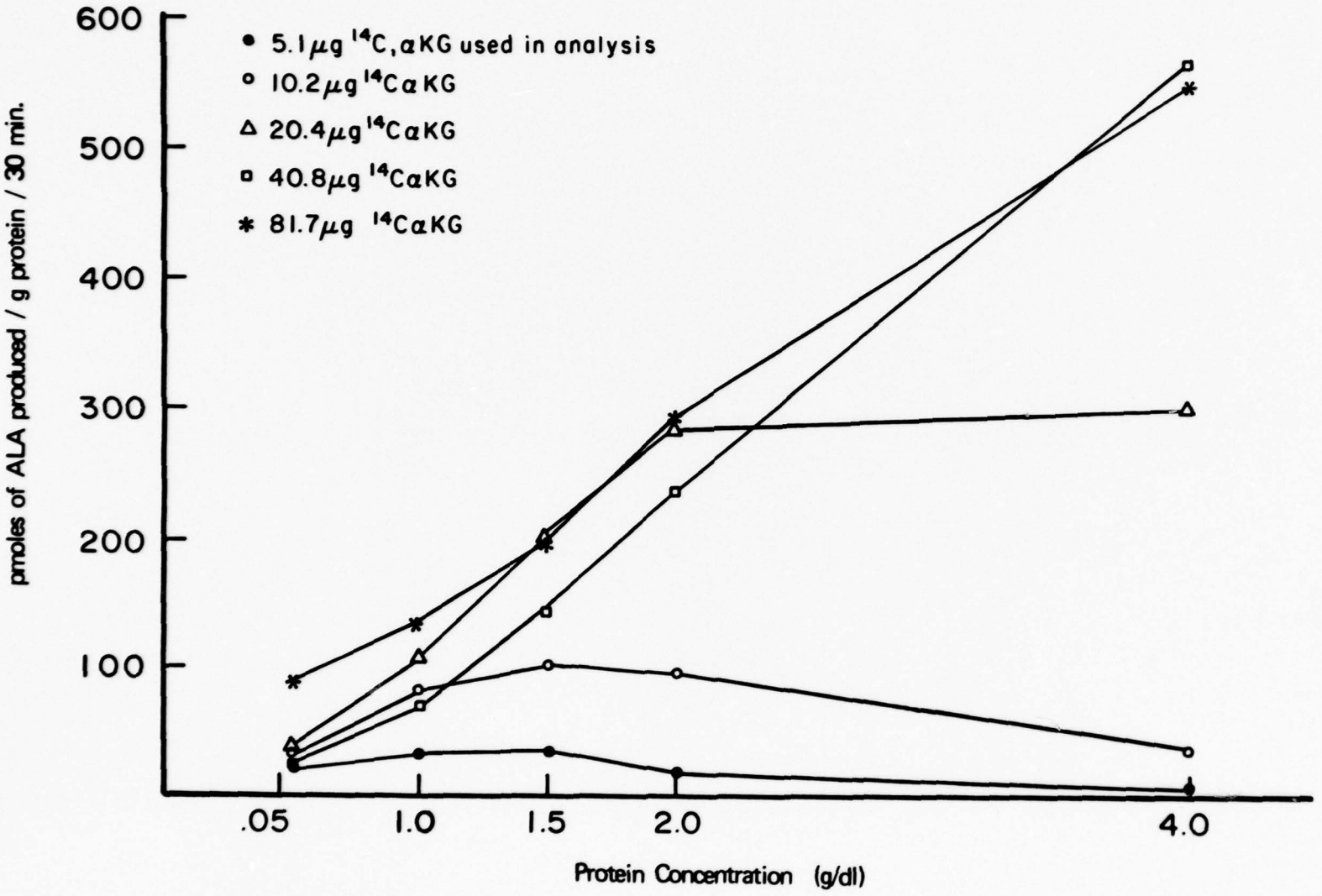


Figure 4. Relationship between Heme Synthetase activity and protein concentration of rat liver homogenate.

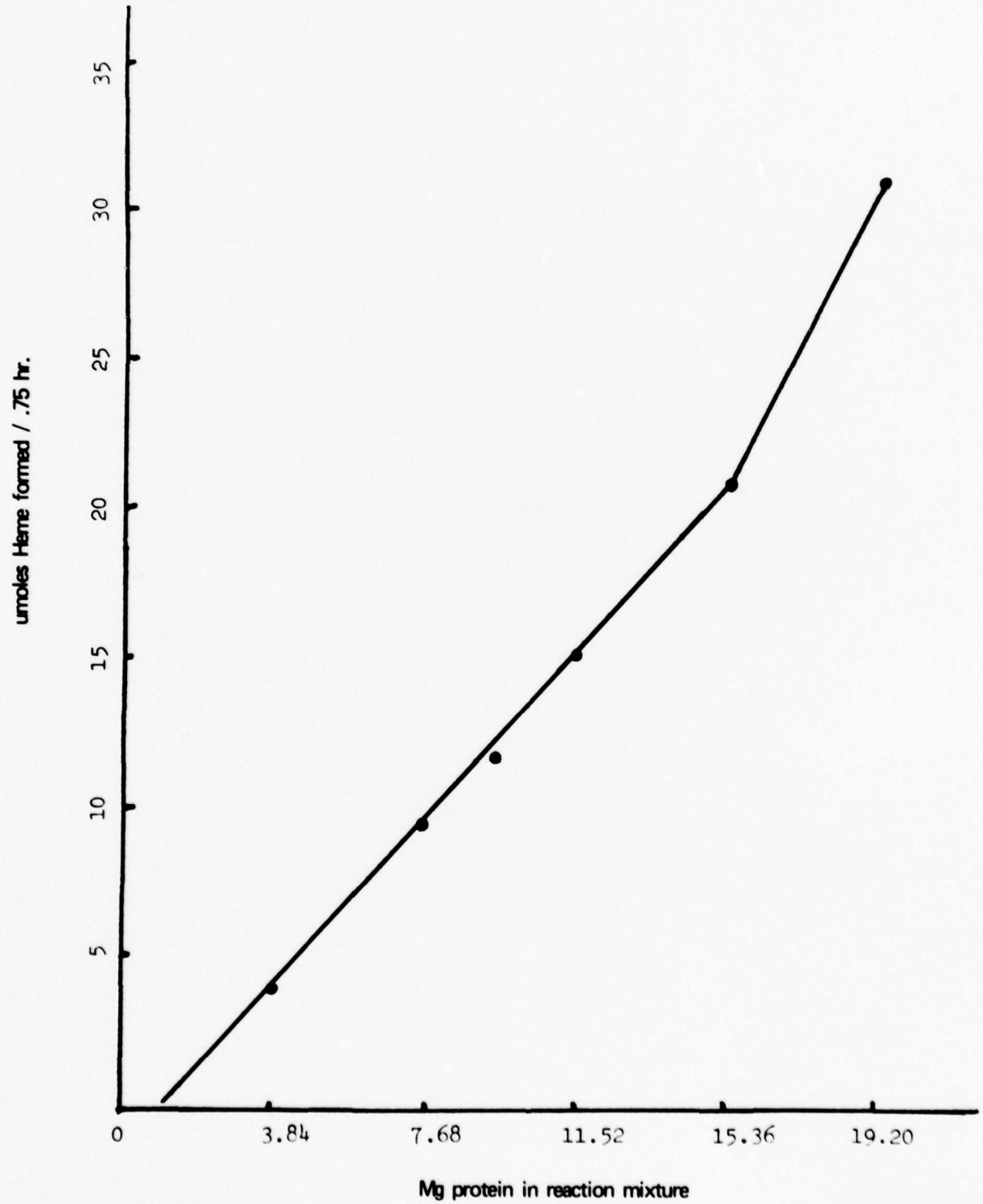


Figure 5. Variation of Heme Synthetase activity with Iron concentration

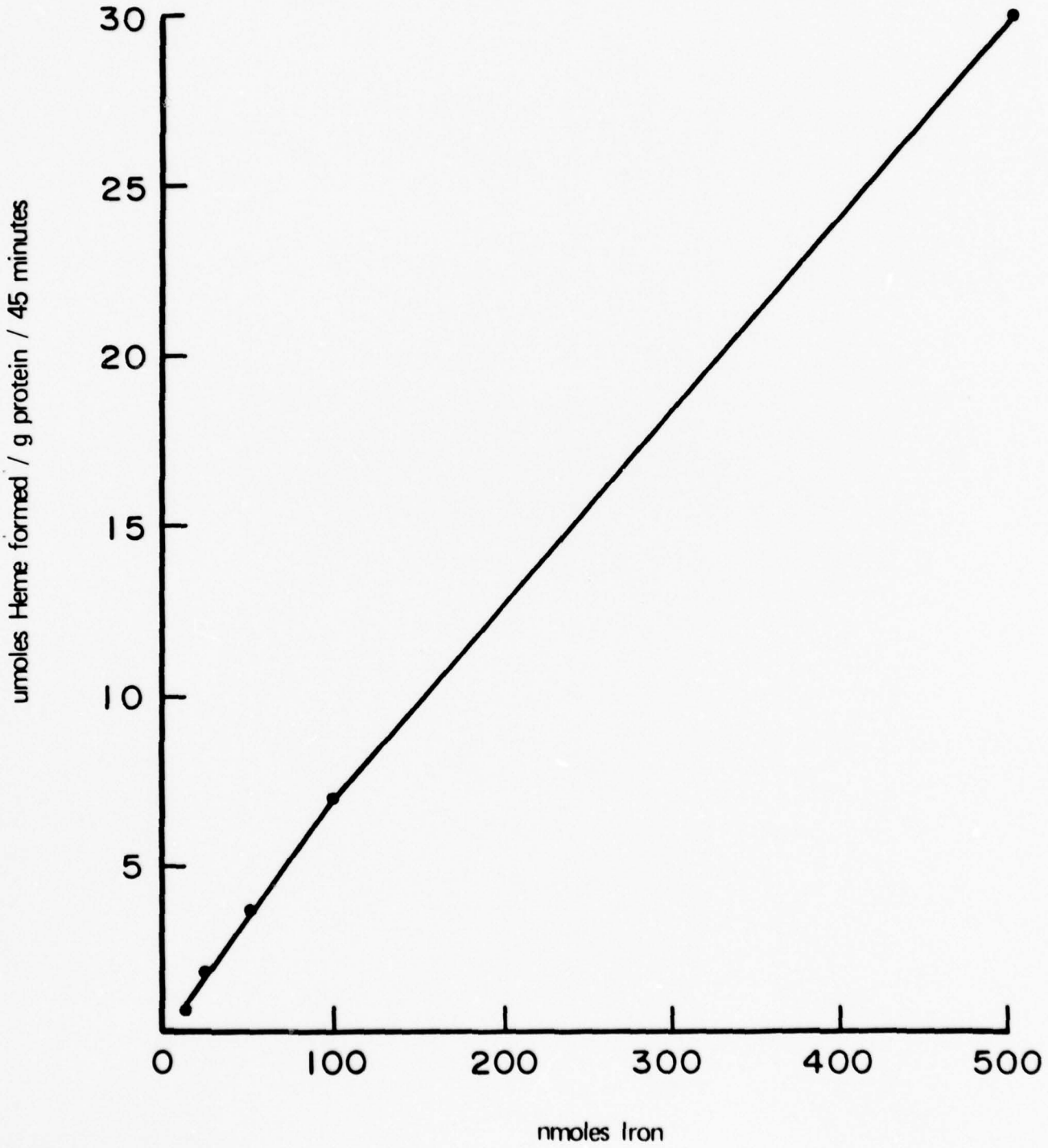


Figure 6. Variation of Heme Synthetase activity with Porphyrin concentration

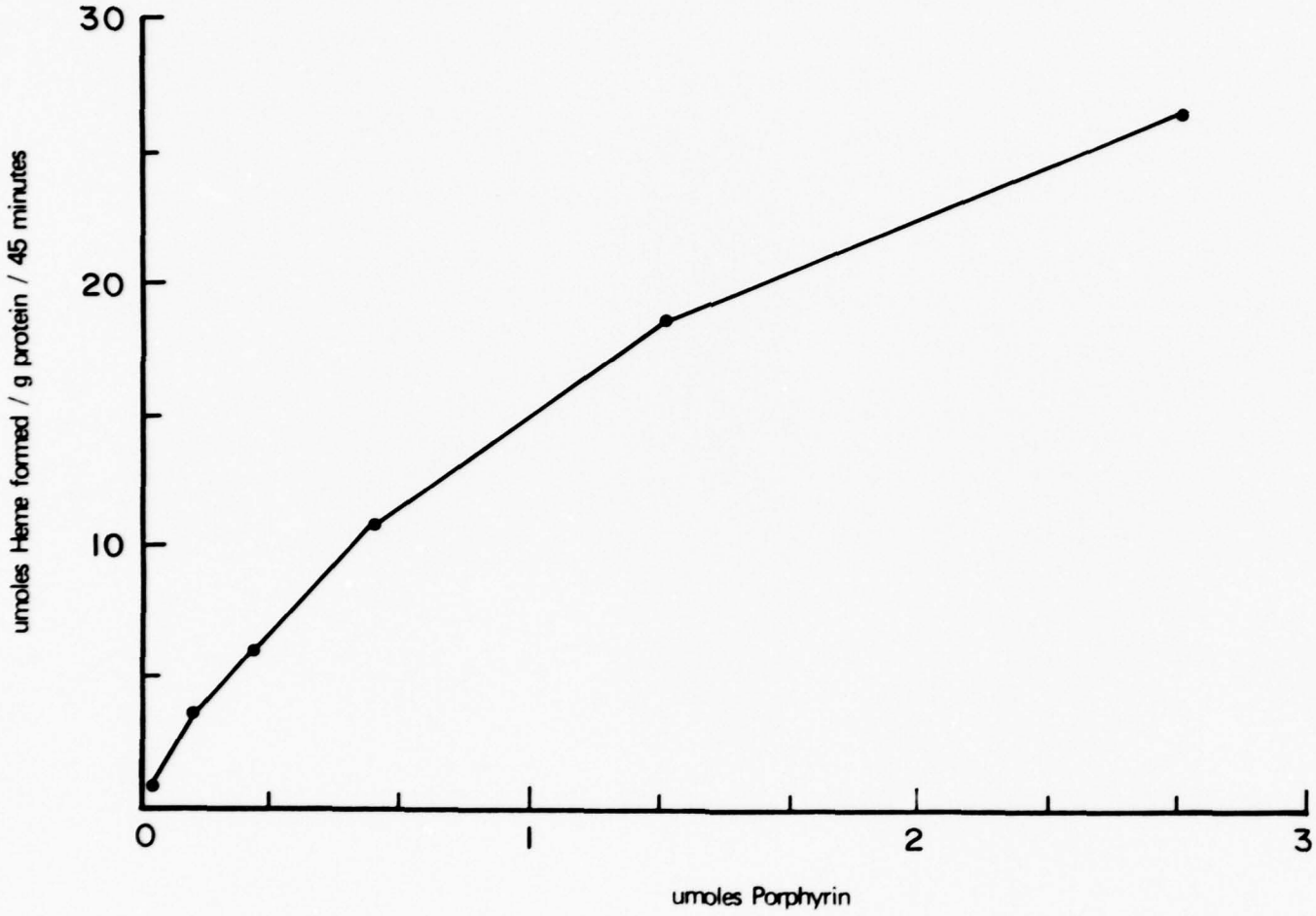


Figure 7. Rate of Cell-Free Incorporation of Radiolabeled Leucine into Protein by Rabbit Reticulocyte Lysate System

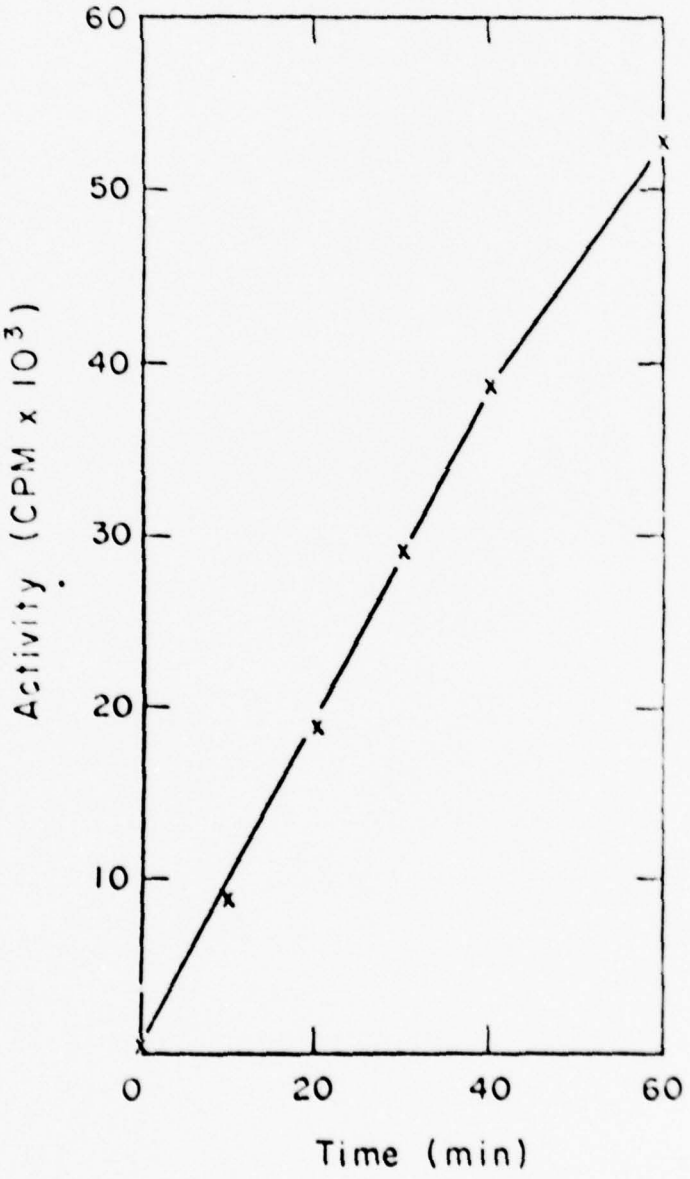
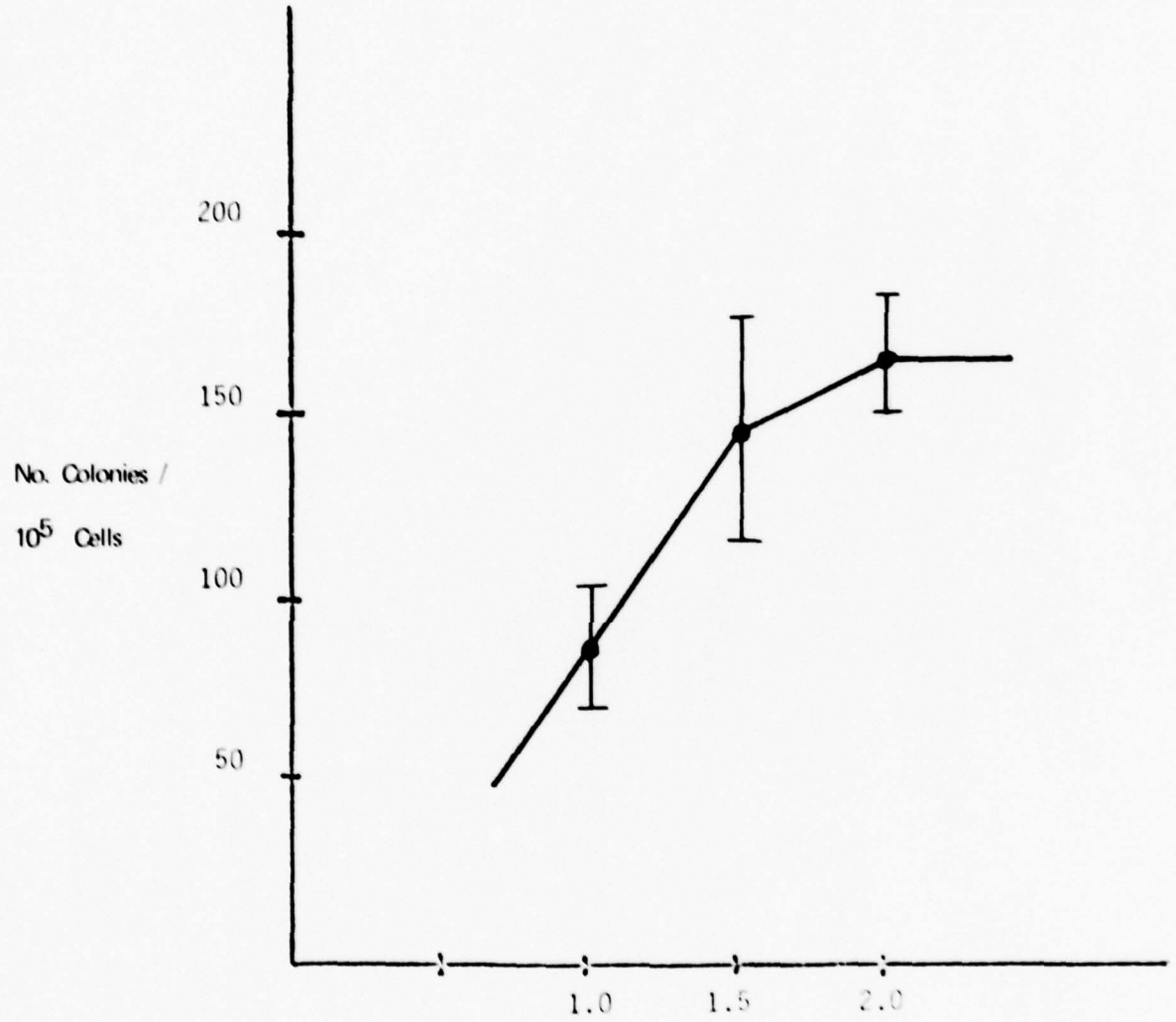


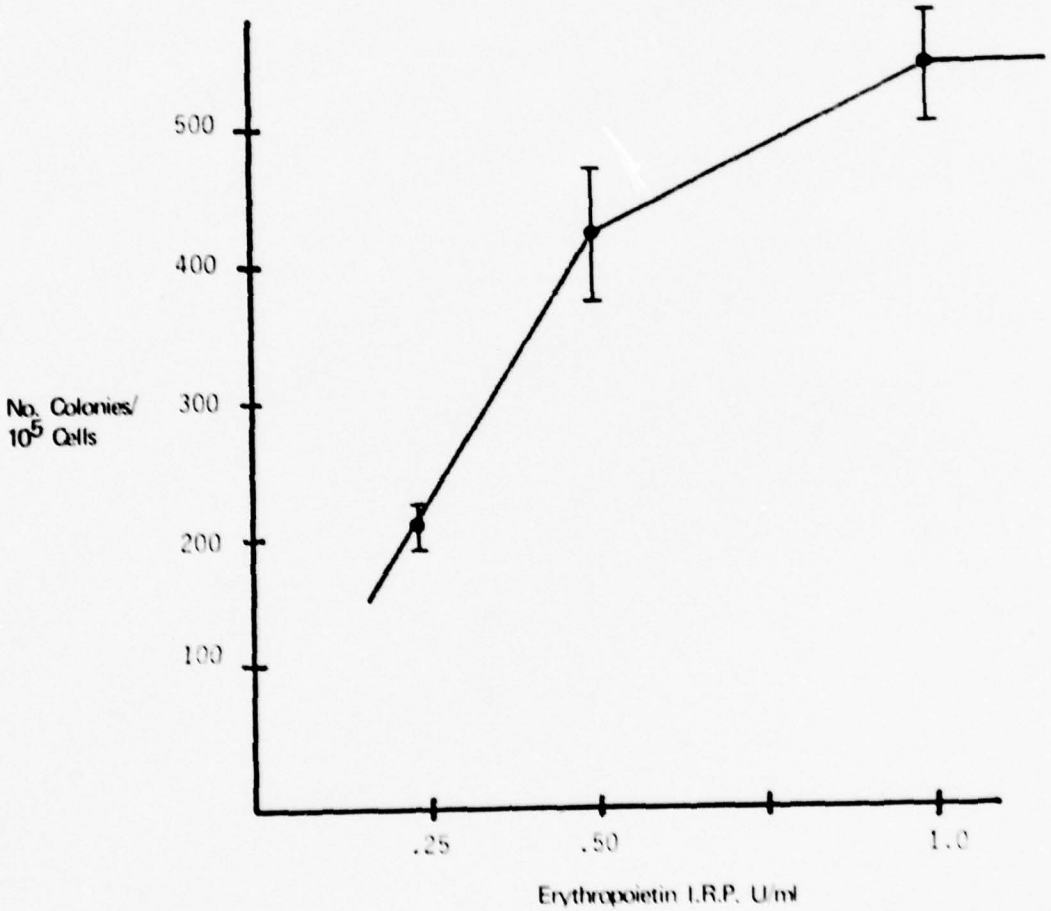
Figure 8. Effect of Initial Inoculum on Colony Numbers



No. of Cells in Initial Inoculum	No. of Colonies / 10^5 Cells
1.0×10^5/ml	84 (+ 14)[a]
1.5×10^5	146 (+ 27)
2.0×10^5	164 (+ 16)

[a] Value = mean (+ S.E.M.) of 3 replicate cultures.

Figure 9. Erythropoietin Dose Response



(Erythropoietin)	No. of colonies/10^5 cells (initial inoculum)
0.25 units/ml	205 (+ 20)[a]
0.50	426 (+ 52)
1.00	550 (+ 42)

Figure 10. Effect of Various Organic Solvents on Cell-Free Synthesis of Rabbit Globin

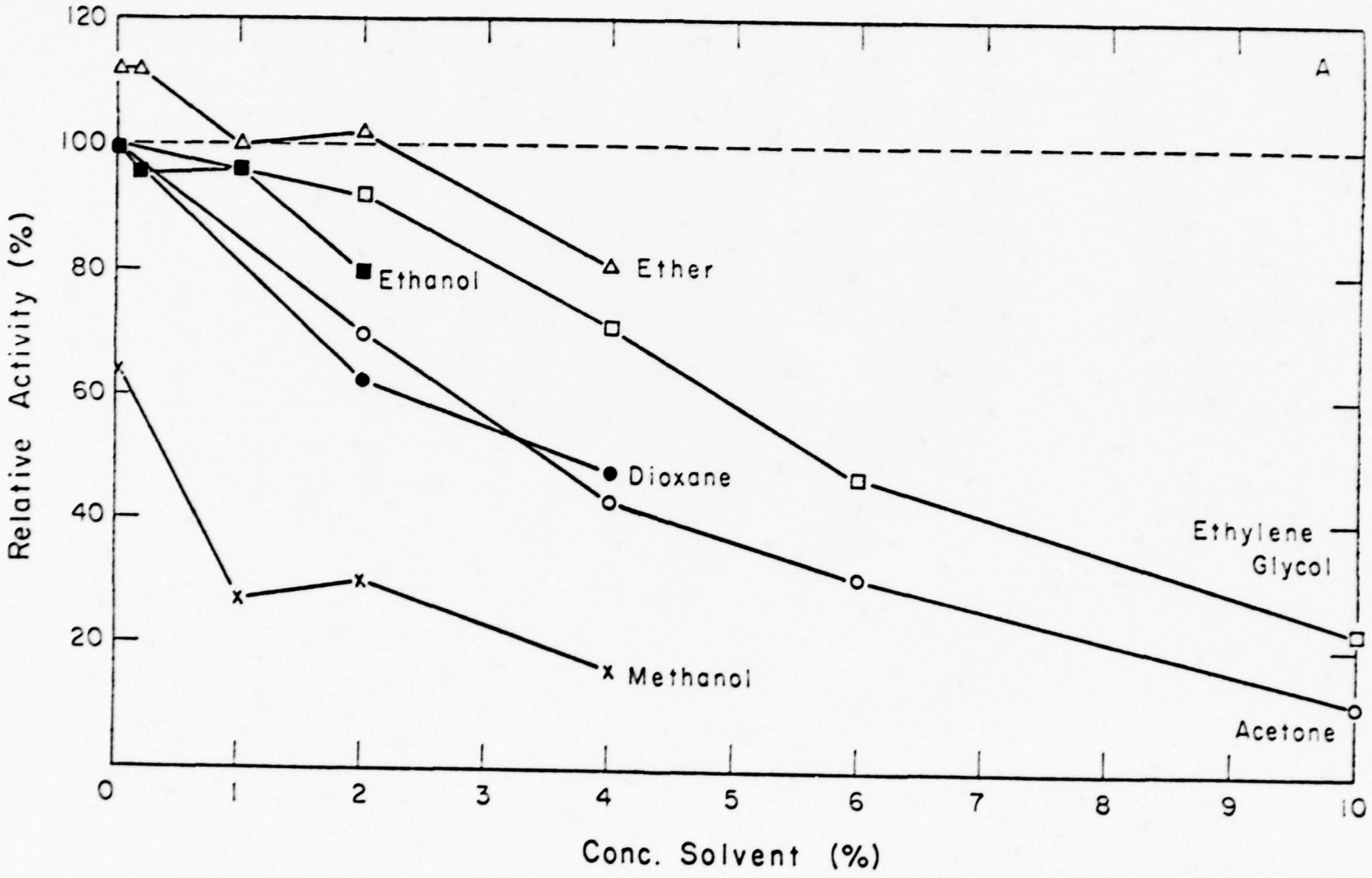


Figure 11. Effect of a Detergent and Detergent-Alcohol Mixture on Reticulocyte Protein Synthesis

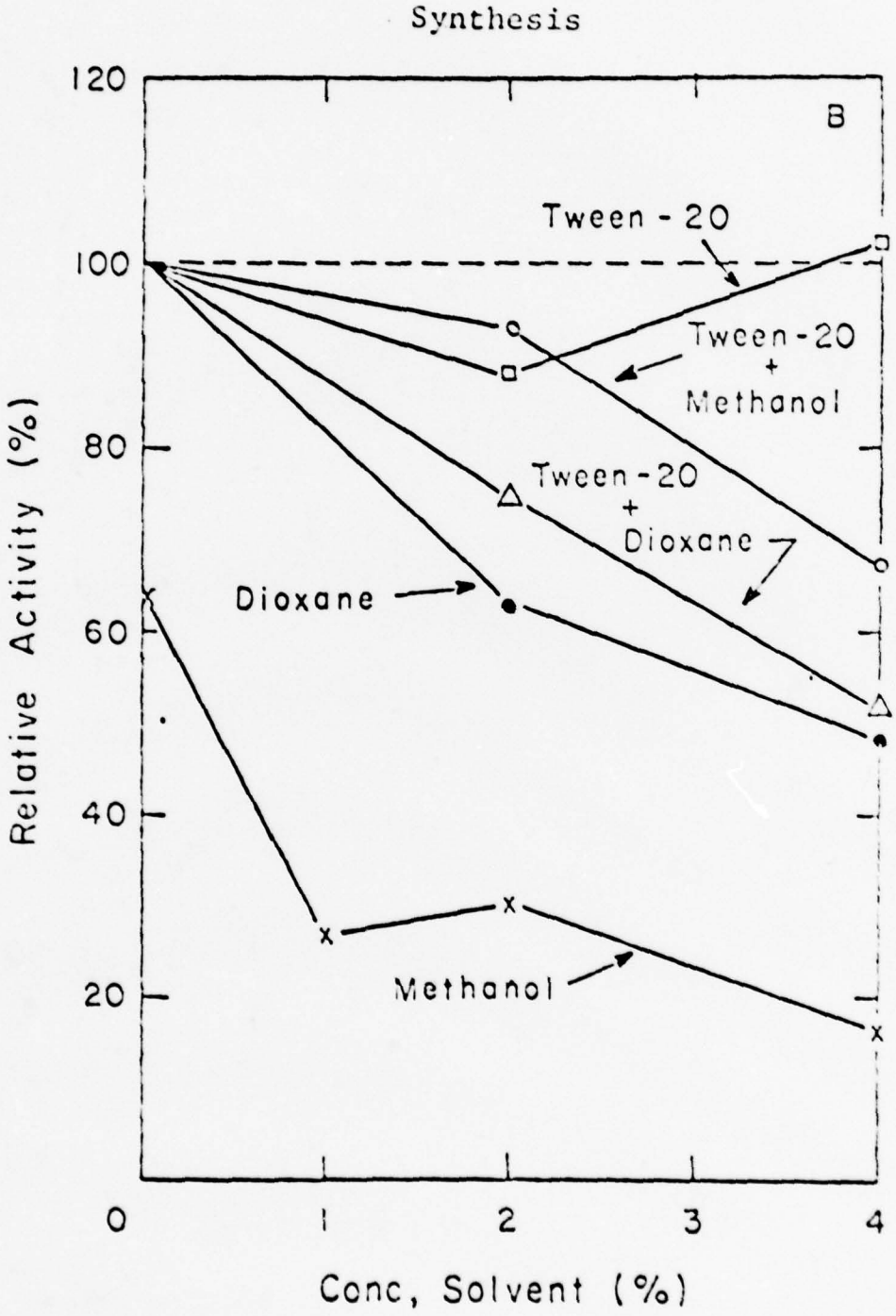


Figure 12. Effect of Benzene on Cell-Free Protein Synthesis by Rabbit Reticulocyte Lysate

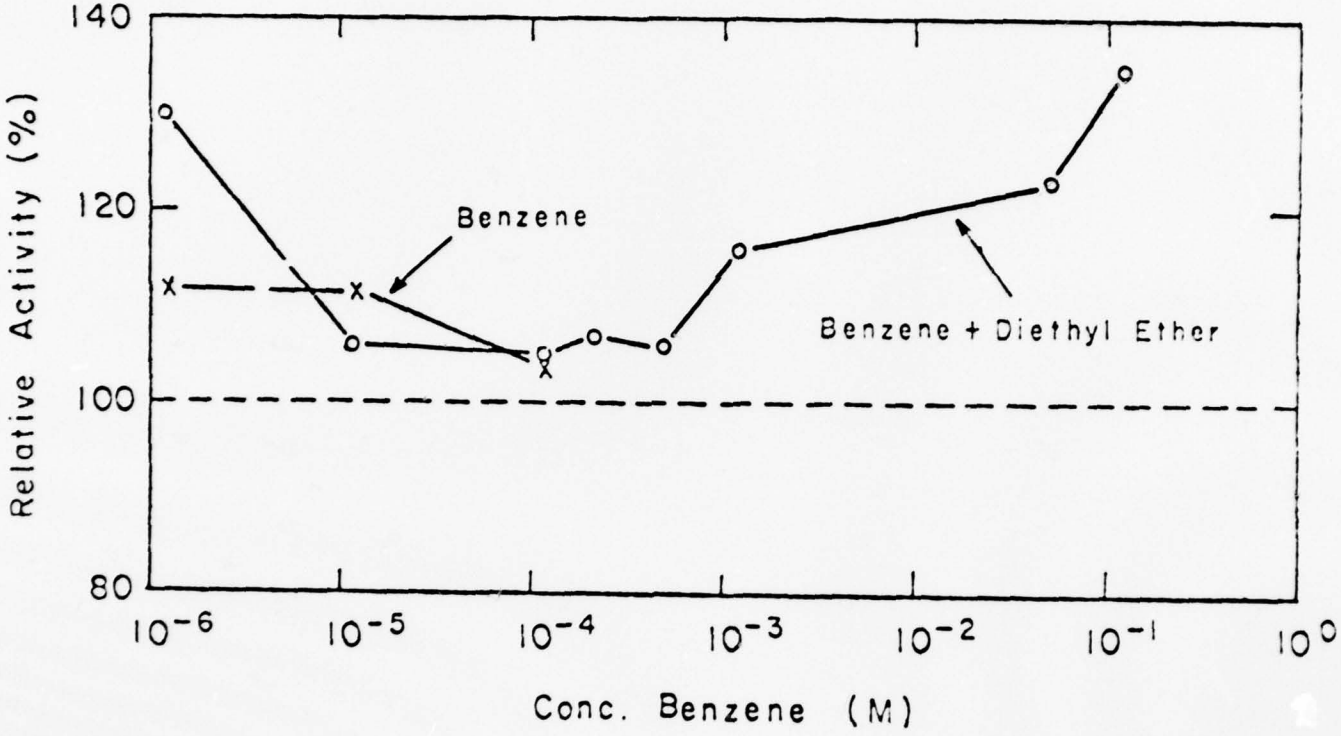


Figure 13

THE DINITROBENZENES

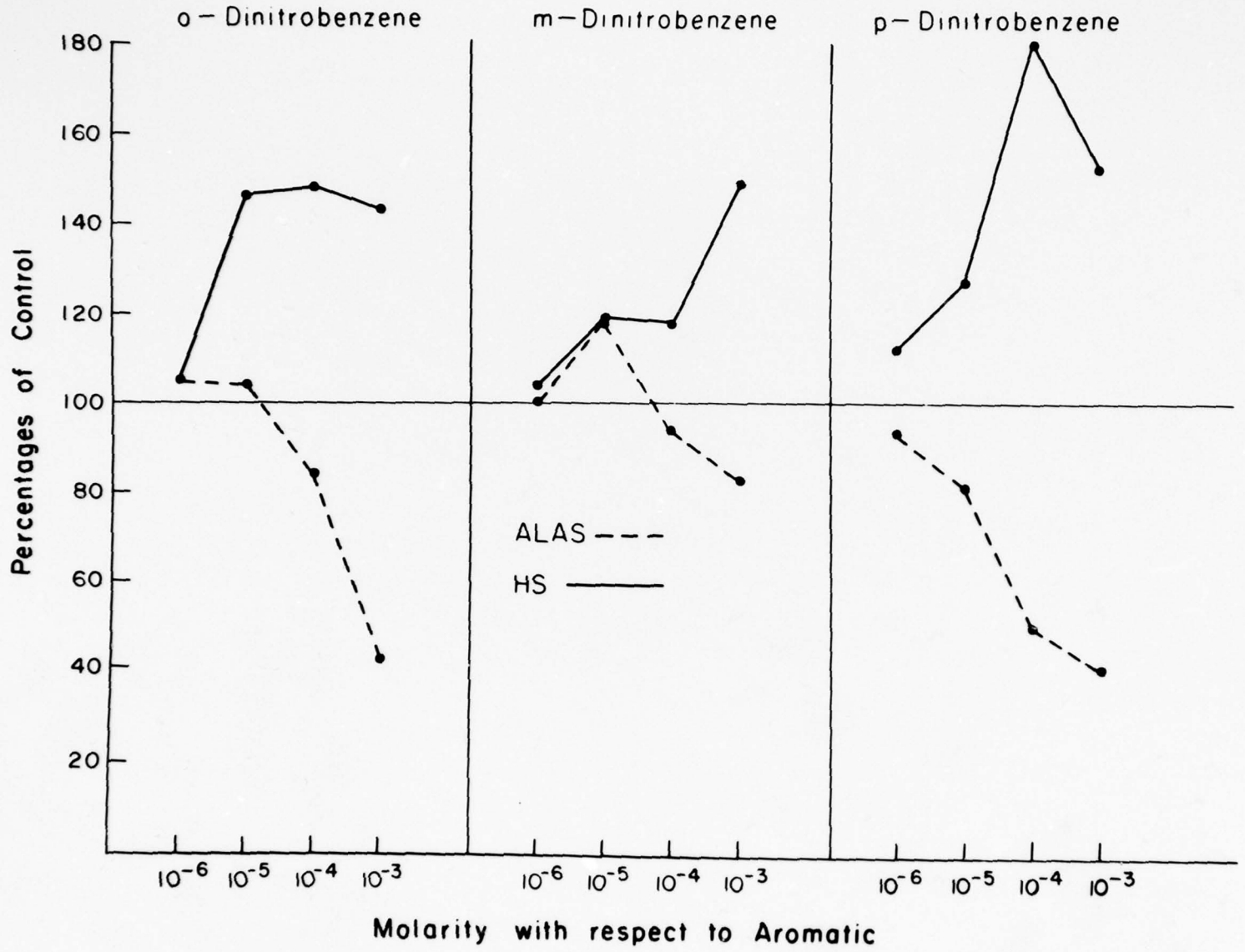


Figure 14. Effect of Three Isomers of Dinitrobenzene (DNB) on Cell-Free Synthesis of Rabbit Globin

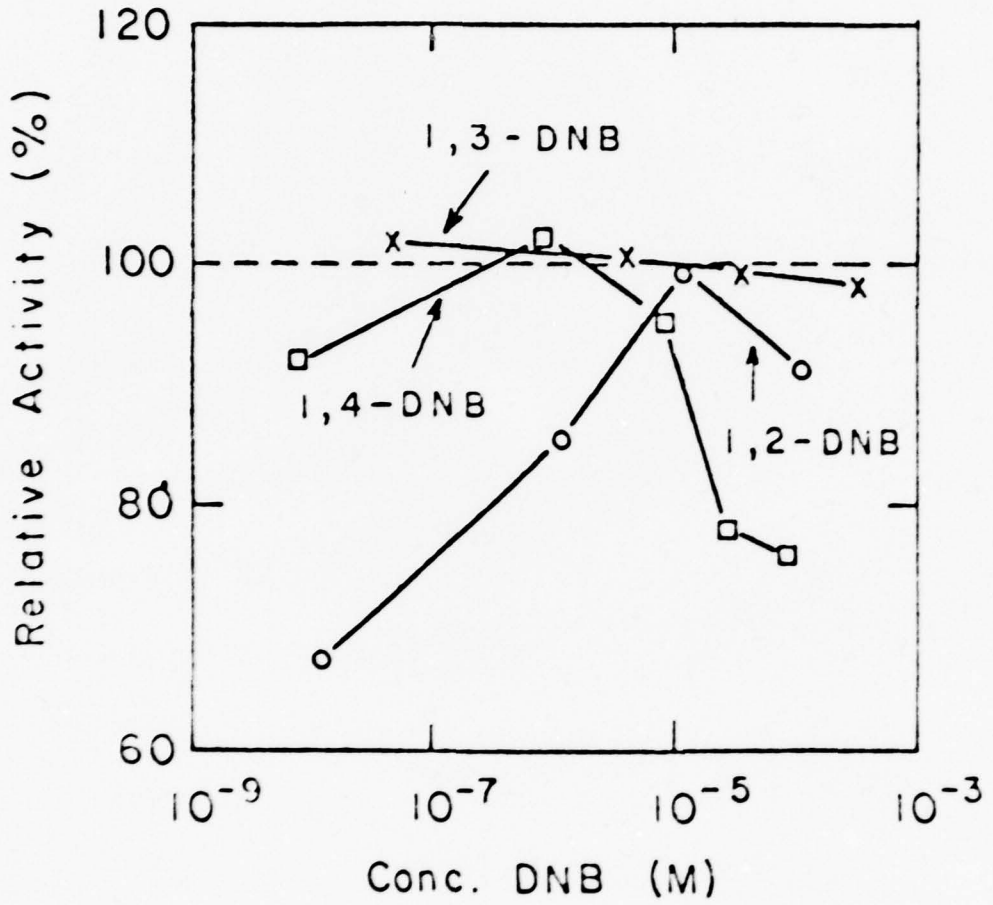


Figure 15

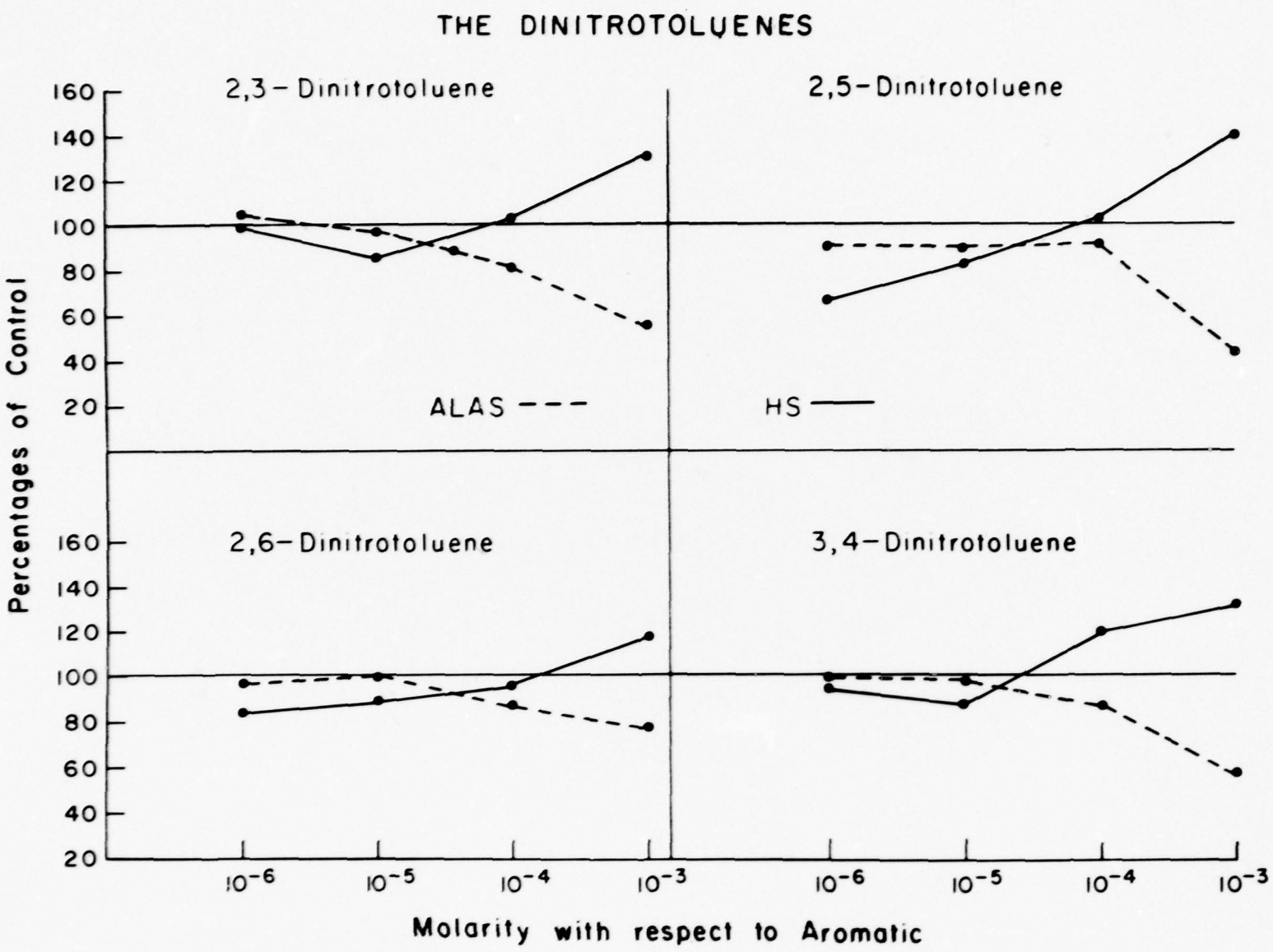

THE DINITROTOLUENES
2,3 – Dinitrotoluene
2,5 – Dinitrotoluene
2,6 – Dinitrotoluene
3,4 – Dinitrotoluene
ALAS ---
HS ——
Percentages of Control
160
140
120
100
80
60
40
20
10^{-6}
10^{-5}
10^{-4}
10^{-3}
Molarity with respect to Aromatic

Figure 16

THE TRI-SUBSTITUTED TOLUENES

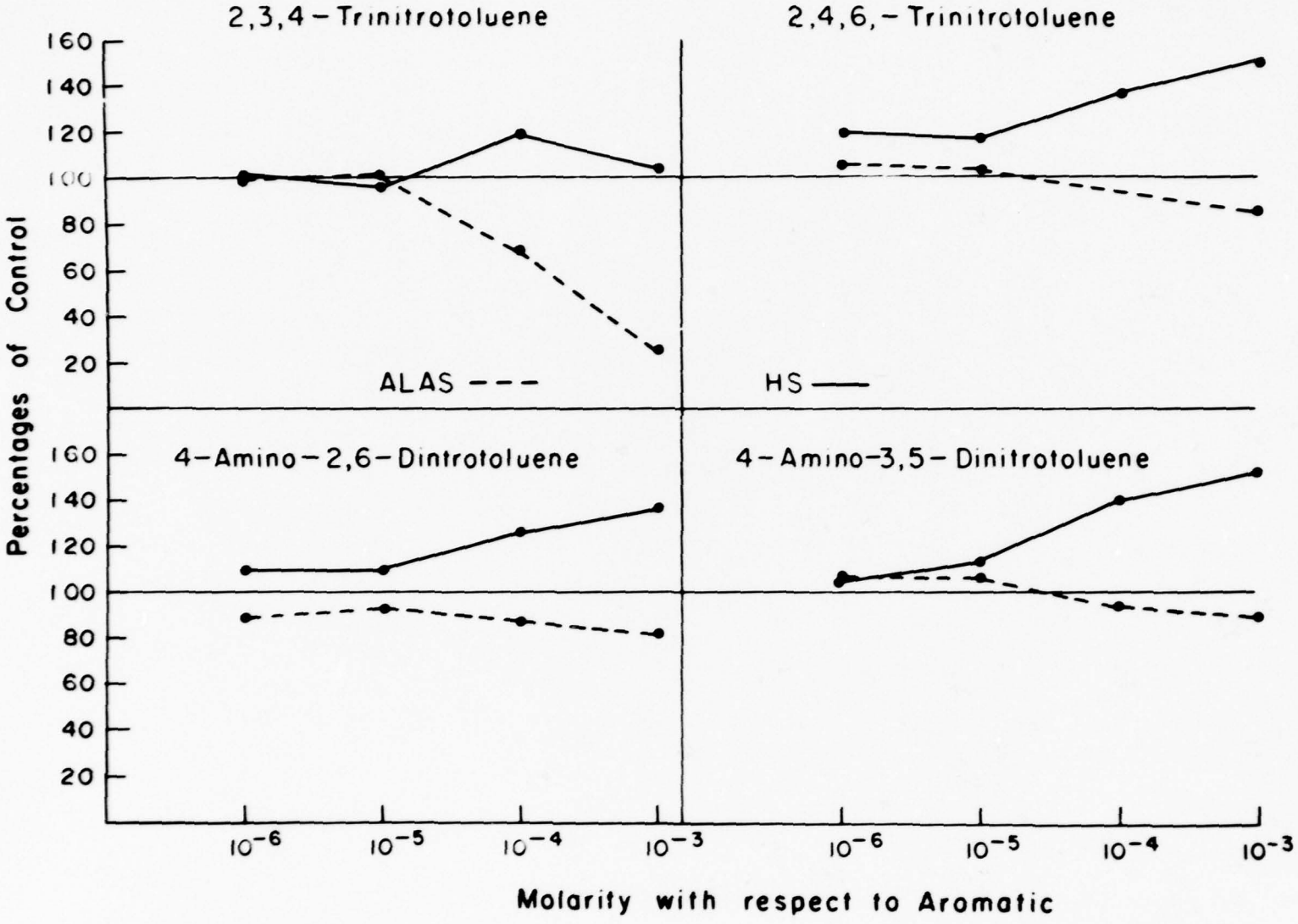


Figure 17. Effect of Various Dinitrotoluene Isomers on In Vitro Protein Synthesis by Rabbit Reticulocyte Lysates

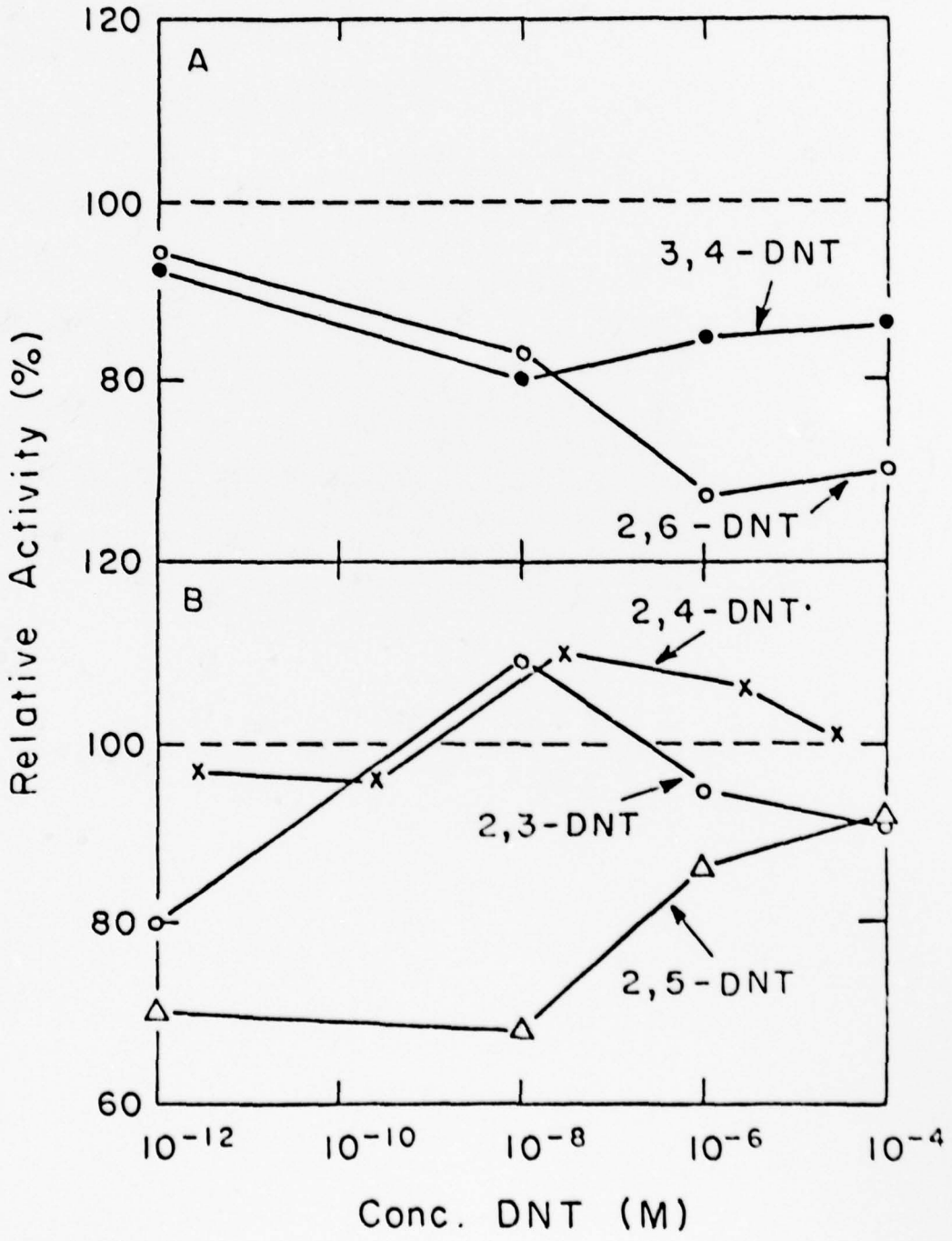
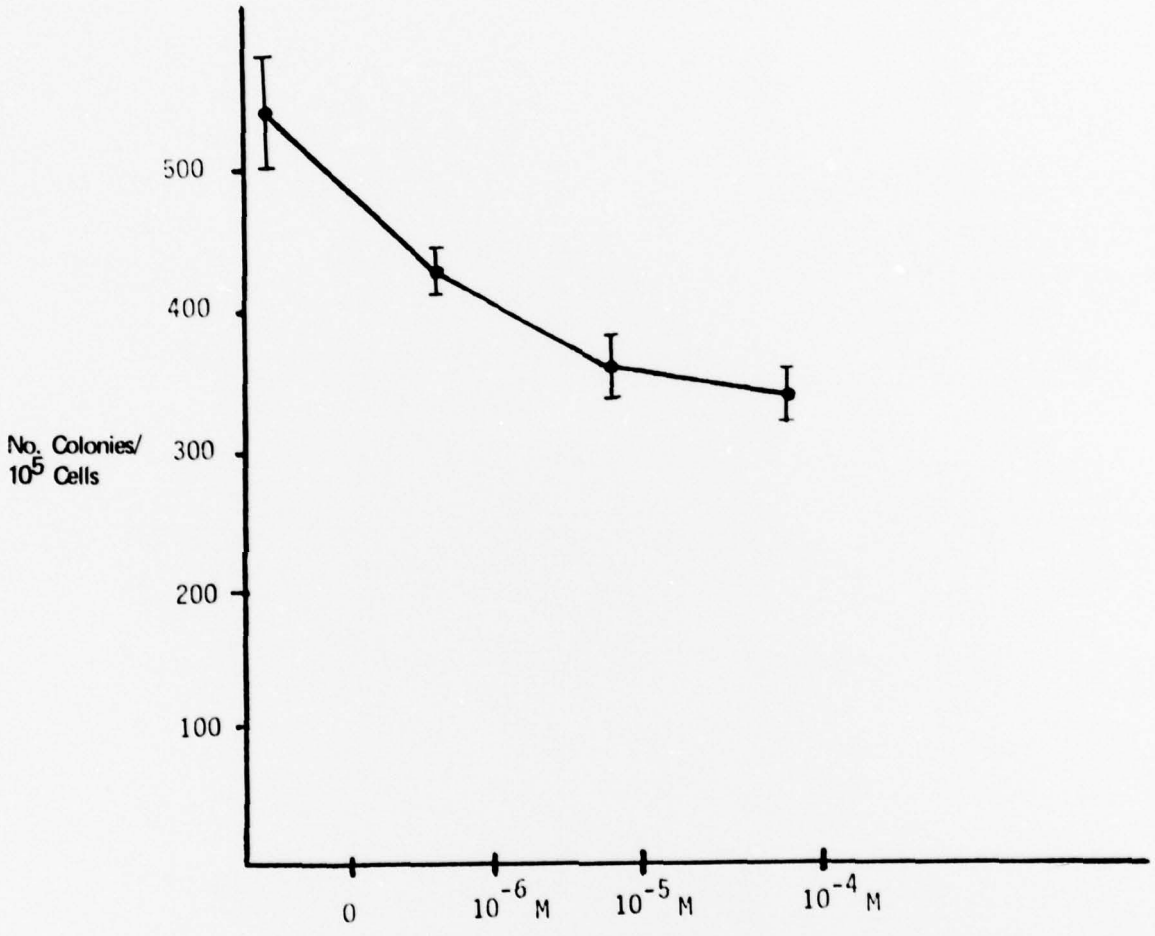


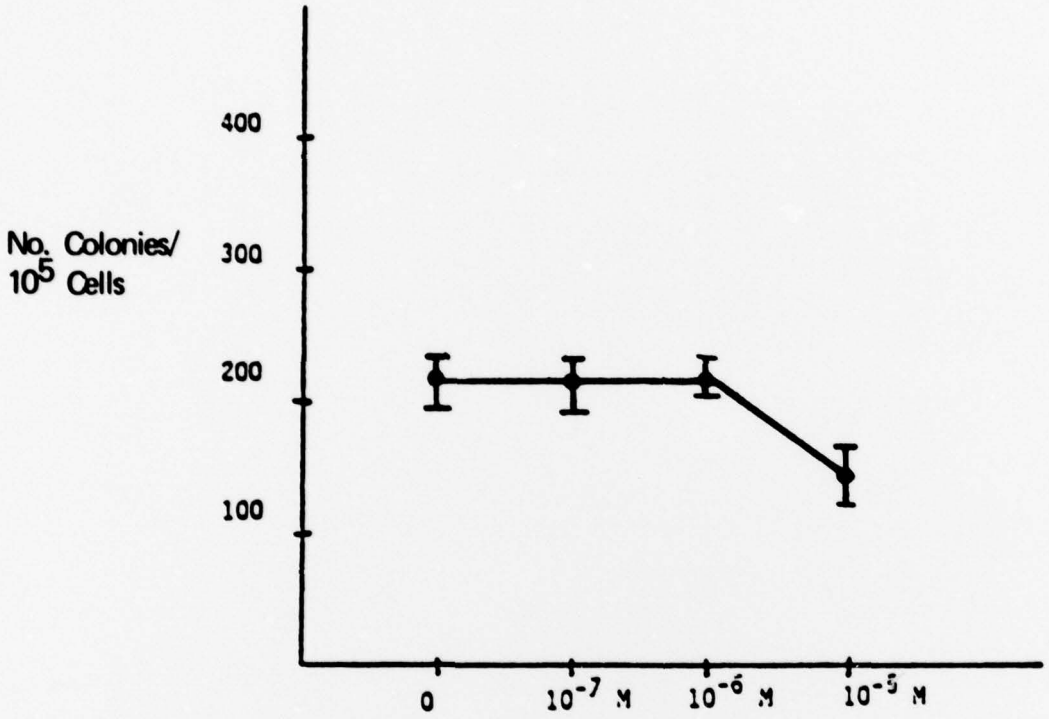
Figure 18. Effect of Concentration of 2,6-DNT on Colony Number



(2,6-DNT)	No. of Colonies/10^5 cells (*initial inoculum*)	
0	550 (± 42)[a]	
10^{-6} M	433 (± 13)	21% inhibition
10^{-5} M	365 (± 15)	34% inhibition
10^{-4} M	339 (± 19)	38% inhibition

[a] Value = mean (± S.E.M.) of 3 replicate cultures.

Figure 19. Effect of Concentration of 3,4-DNT on Colony Number



final (3,4-DNT)	No./10^5	
	217 (± 19)[a]	
10^{-7} M	215 (± 19)	
10^{-6} M	217 (± 9)	
10^{-5} M	145 (± 23)	33% inhibition

[a] Value = mean (± S.E.M.) of three replicate cultures.

Table I. Day-to-day and within-day variation of ALAsynthetase activity on pooled rat liver homogenate-sonicates. Aliquots of pooled homogenate were frozen at -70°C and examined at days 2, 3, 7, and 20.

	Day 0	Day 2	Day 3	Day 7	Day 20
nmol / g protein per 0.5 hr.	3.70	3.79	3.80	3.77	3.49
	3.65	3.81	3.74	3.86	3.30
	3.63	3.63	3.55	3.84	3.30
	3.63	3.61	3.71	3.88	3.46
	3.63	3.64	3.70	3.69	3.45
	3.63	3.61	3.63	3.83	3.31
	3.63	3.67	3.89	3.72	3.25
	3.64	3.78	3.72	3.82	3.33
	3.61	3.68	3.53	3.88	3.32
	3.60	3.69	3.69	3.99	3.28
Mean	3.64	3.69	3.70	3.83	3.37
S.E.M.	.009	.024	.034	.027	.029

Table II. Within-day reproducibility of heme synthetase. Homogenate-sonicates from seven individual livers were examined.

	Homogenate						
	1	2	3	4	5	6	7
umol Heme formed per 45 min. per g protein	17.8	13.6	11.9	21.3	20.1	22.9	13.3
	16.2	11.4	12.9	27.9	22.0	19.2	13.2
	17.2	11.9	12.6	26.1	21.7	23.0	12.5
		12.1	12.2	25.3	21.8	23.3	
		12.5	13.8	27.3	20.0		
		12.0	11.7	24.1			
			12.4				
			11.7				
Mean	17.1	12.3	12.4	25.3	21.1	22.1	13.0
S.E.M.	.47	.31	.25	.98	.44	.97	.25

Table III.

STABILITY OF HEME SYNTHETASE

	umol / g protein / 45 min.			
RAT 1:	Day 1	Day 24		Day 58
	15.5	14.9		10.9
	17.3	18.2		12.4
Mean:	16.4	16.7		11.7
RAT 2:	Day 1	Day 25	Day 27	Day 28
	11.7	11.9	11.6	11.1
	10.3	11.6	12.0	10.6
		11.2		
Mean:	11.0	11.6	11.8	10.9

TABLE IV. Effect of Added Heme on Heme Synthetase Activity

nmol of Added Heme	umol of Heme formed/g/45 min.
0	40.1
4	39.7
8	38.4
20	33.4
40	27.4
80	36.9
160	31.1
200	28.5

Table V

Cell-Free Synthesis of Protein by Five Different Preparations of Rabbit Reticulocytes

Lysate	^{3}H-Leucine Incorporation (p moles x 10^{2})
1	2.3
2	2.8
3	15.0
4	2.8
5	2.7

Activity is expressed as pmols leucine incorporated into protein per 50 ul reaction mixture (20 ul lysate) after one hour incubation.

Table VI

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

Solvent	Relative Activity (%)					
	.2%	1%	2%	4%	6%	10%
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 VII

Effect of Benzene on Cell-Free Protein Synthesis by Rabbit Reticulocyte Lysate

Conc. of Benzene (M)	Relative Activity (%) in Diethyl Ether	in H_2O
1.78×10^{-1}	135	-
7.12×10^{-2}	123	-
1.78×10^{-2}	116	-
7.12×10^{-3}	106	-
3.56×10^{-3}	107	-
1.78×10^{-3}	105	104
1.78×10^{-4}	106	112
1.78×10^{-6}	130	112

Table VIII

Effect of Three Isomers of Dinitrobenzene (DNB) on Cell-Free Synthesis of Rabbit Globin

Conc. Dinitrobenzene (M)	Relative Activity (%) 1,2-DNB	1,3-DNB	1,4-DNB
5.9×10^{-4}	-	98	-
1.8×10^{-4}	91	-	-
9.4×10^{-5}	-	-	76
5.9×10^{-5}	-	99	-
4.7×10^{-5}	-	-	78
1.8×10^{-5}	99	-	-
9.4×10^{-6}	-	-	95
5.9×10^{-6}	-	100	-
1.8×10^{-6}	85	-	-
9.4×10^{-7}	-	-	102
5.9×10^{-8}	-	102	-
1.8×10^{-8}	67	-	-
9.4×10^{-9}	-	-	92

Table IX

Effect of Various Dinitrotoluene Isomers on in vitro Protein Synthesis by Rabbit Reticulocyte Lysates

Conc. of Dinitrotoluene	2,3-DNT	2,5-DNT	2,6-DNT	3,4-DNT	2,4-DNT[+]
1×10^{-4}	91	92	70	86	-
4.6×10^{-5}	-	-	-	-	101
4.6×10^{-6}	-	-	-	-	106
1×10^{-6}	94	86	67	84	-
4.6×10^{-8}	-	-	-	-	110
1×10^{-8}	109	68	83	80	-
4.6×10^{-10}	-	-	-	-	96
4.6×10^{-12}	-	-	-	-	-
1×10^{-12}	80	70	94	92	97

[+] 2,4-DNT was solubilized in H_2O, all other isomers in ethanol.

Table X.

COMPARISON OF TOXICITY STUDIES

Compound	96 hr. LC50 (mg / liter) in Minnows	ALAS (Percentage of Control)	HS
Toluene	12.6	96	92
4-Nitrotoluene	49.9	84	93
2,3-Dinitrotoluene	1.9	55	130
2,4-Dinitrotoluene	32.5	92	87
2,5-Dinitrotoluene	1.3	43	139
2,6-Dinitrotoluene	19.8	77	117
3,4-Dinitrotoluene	1.5	56	131
2,4,6-Trinitrotoluene	2.4	86	150
2-Amino-4-Nitrotoluene	71.3	89	97
4-Amino-2,6-Dinitrotoluene	6.9	82	137
4-Amino-3,5-Dinitrotoluene	13.1	89	152

NOTE: The lower the LC50 number obtained by Ft. Detrick, the more drastic the change in the percentage of control we obtained for ALAS (usually a decrease) and for HS (usually an increase).

Table XI.

Concentration of Aromatic	Percentages of Control							
	10^{-3} mol/l		10^{-4} mol/l		10^{-5} mol/l		10^{-6} mol/l	
Test	ALAS	HS	ALAS	HS	ALAS	HS	ALAS	HS
Aromatic								
Benzene	95	86	104	91	107	94	107	89
Toluene	96	92	104	86	93	91	100	89
Nitrobenzene	86	111	108	104	110	100	81	105
o Dinitrobenzene	43	143	84	148	104	146	105	105
m Dinitrobenzene	83	149	94	118	118	119	100	104
p Dinitrobenzene	41	152	50	180	81	127	93	112
4 Nitrotoluene	84	93	95	99	96	107	96	113
2,3 Dinitrotoluene	55	130	81	103	97	85	104	99
2,4 Dinitrotoluene	92	87	100	80	102	92	99	96
2,5 Dinitrotoluene	43	139	91	102	89	82	90	66
2,6 Dinitrotoluene	77	117	87	96	100	89	97	84
3,4 Dinitrotoluene	56	131	86	119	97	87	99	94
2,3,4 Trinitrotoluene	26	104	69	119	101	96	99	100
2,4,6 Trinitrotoluene	86	150	93	136	103	117	105	119
4 Amino 2,6 Dinitrotoluene	82	137	87	126	93	110	89	109
4 Amino 3,5 Dinitrotoluene	89	152	94	140	107	113	107	105
2 Amino 4 Nitrotoluene	89	97	88	111	87	118	94	103
o Chlorotoluene	87	101	98	118	99	108	94	111
m Chlorotoluene	97	107	99	110	101	104	103	104
p Chlorotoluene	97	108	100	108	102	106	103	104
1 Chloro 2 Nitrobenzene	102	115	88	113	95	109	99	110
Hexachlorobenzene	117	133	94	131	99	116	104	113
Aniline	96	108	91	121	94	104	98	116

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