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REPORT NO. 3

**STUDIES OF BIOLOGICALLY ACTIVE AGENTS
IN CELLS AND TISSUE CULTURES**

PART 1

ANNUAL PROGRESS REPORT

by

JANIS GABLIKS, Ph.D.

DECEMBER 1966

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Massachusetts Institute of Technology
Department of Nutrition and Food Science
Cambridge, Massachusetts 02139

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ANNUAL PROGRESS REPORT
JANUARY 1966 - DECEMBER 1966

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STUDIES OF BIOLOGICALLY ACTIVE AGENTS IN
CELL AND TISSUE CULTURES
(PART I)

CONTRACT NO. DA-49-193-MD-2533

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SUMMARY

1. Prepared by: Department of Nutrition and Food Science
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139
 2. Title of Report: Studies of Biologically Active Agents in
Cell and Tissue Cultures, Part I
Report No. 3, December 1966
 3. Principal Investigator: Dr. Janis Gabliks
Assistant Professor of Cell Biology
 4. 41 pages, 13 figures
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I. TOXINS PRODUCED BY MICROORGANISMS

Staphylococcal enterotoxin B is cytotoxic to a cell strain derived from human embryonic intestine. The susceptibility of cells to the toxin was markedly reduced by trypsin. The temporary resistance increased proportionally with increasing time of contact of the cells with trypsin and lasted for 48 hours. The effect is analogous to the trypsin-induced resistance of HeLa cells to polio and coxsackie viruses and suggests that trypsin inactivates specific cell receptors which may be essential for interaction with enterotoxin.

II. SELECTED TOXIC SUBSTANCES

Insecticidal compounds DDT, chlordane, Kelthane^R, Dipterex^R, malathion, and Karathane^R at subtoxic concentrations inhibited vaccinia virus replication in human Chang liver cells. Under the same experimental conditions, the replication of poliovirus was inhibited only by chlordane and malathion, whereas Kelthane and Karathane increased the virus yields 4 and 18 times, respectively, and DDT exhibited a slight stimulatory effect.

Since the reduction in virus yields was not due to extracellular inactivation of virus, the results suggest that some insecticides affect virus replication by altering some physiological activities of cells. Consequently, the magnitude of virus replication may be useful as a parameter for the detection of toxicity of chemicals below their acute toxicity levels.

III. NUTRITIONAL FACTORS IN VIRAL INFECTIONS

Studies on the interrelationship between the nutritional state of hosts and virus diseases are concerned with the effects of a single amino acid deficiency on virus replication. A model system in cell cultures has been developed for simultaneous evaluation of the effects of essential nutrients on cell growth and on virus replication.

A deficiency of methionine and glycine, induced by their analogues, and a deficiency of leucine in the medium suppressed vaccinia virus replication significantly more than they inhibited the growth of cells. The replication of virus was also inhibited when the medium contained an excess of either methionine, glycine, leucine, isoleucine or valine. The inhibitory effect of methionine on virus replication in cell cultures correlated well with the results obtained with vaccinia virus infection in the rabbit.

7. Key words: Cell Cultures
Staphylococcal Enterotoxin B
Diphtheria Toxin
Insecticides
Organophosphorus Compounds
Amino Acids
Virus Infections

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INTRODUCTION

The studies conducted under the provisions of the research contract were directed towards developing sensitive methods for the detection of physiological and pathological changes in cultivated cells, to study the mode of action of toxic substances at the cellular level, and to investigate possible means for the therapeutic counteraction of harmful agents.

This report presents summaries of the results obtained in three major areas of investigations:

- I. Toxins produced by microorganisms
- II. Selected toxic substances--insecticidal compounds
- III. Nutritional factors in viral infections.

The investigations were carried out by the principal investigator--Assistant Professor J. Gabliks (Ph.D.) and Research Associate W. Schaeffer (Ph.D.) with assistants: M. Falconer (B.S.), R. Calitis (B.S.), G. Strachan (M.S.), M. Bantug (M.S.), L. Anthony (B.S.), and O. Baralt.

Herewith we acknowledge the help of Dr. Nevin Scrimshaw, Professor of Nutrition and of Dr. Leo Friedman, Professor of Food Toxicology, for advice and helpful criticism during various phases of this study.

Based on this research contract, seven reports have been published, two are in press and three are being prepared for publication.

I. TOXINS PRODUCED BY MICROORGANISMS

1. Staphylococcal Enterotoxin B

In the previous report we described the cytotoxic

manifestations of enterotoxin B upon cell cultures (1). The data on changes in cell susceptibility showed that the cells of human embryonic intestine (Henle strain) were temporarily resistant to the lethal action of enterotoxin B when challenged with the toxin immediately after planting the cultures, that is, after the ordinary trypsinization procedure which is used to disperse cell monolayers in stock cultures (2). The acquired resistance was maintained for up to 48 hours after trypsinization; however, if the same cell cultures were grown for 48 hours in the absence of toxin after trypsinization and then were challenged with toxin, full susceptibility was evident.

We have continued investigations of this phenomenon. The present report describes the nature of the interaction of enterotoxin with trypsinized cells.

Figure 1 illustrates the results obtained when 100 $\mu\text{g}/\text{ml}$ of Staphylococcal Enterotoxin B (approximately two times the ID_{50} level) was added to cell cultures of human embryonic intestine at the time of planting. It is evident that no growth inhibition or cell destruction takes place as it normally would when this toxin concentration is used on two-day-old cell monolayers.

To evaluate the observed changes in cell susceptibility immediately after the planting of cells from stock cultures, the following three methods were employed to disperse cell monolayers: chemical - methods using trypsin and Versene (ethylenediaminetetraacetic acid) and mechanical - a method for scraping cell monolayers with a "rubber policeman".

Trypsin: To test the effect of trypsin on the response of human intestine cells to enterotoxin B, a solution of 0.042% trypsin (GIBCO) was used on three-day-old cell monolayers in tube cultures. After the trypsin was decanted, the tubes, containing only residual trypsin, were incubated at 37°C for varying time intervals. At the end of incubation one ml of Eagle's basal medium was added to each tube, the cells were dispersed by trituration, and 100 μg enterotoxin was added. The amount of cell protein and cytotoxicity based on morphological changes was measured at 24 and 48 hours. The gradual increase in resistance to the toxin with increase in time of contact of the cells with trypsin is shown in Table 1. The results suggest the enzymatic effect of trypsin, since heat-inactivated trypsin used in a similar test neither removed the cells from the glass surface nor made them resistant to the enterotoxin.

To determine whether cells become permanently resistant to enterotoxin B, 100 $\mu\text{g}/\text{ml}$ of toxin was added to the cell cultures at the time of planting and again 48 hours later. As a control for the second dose of toxin, we used cultures grown 48 hours in the absence of toxin. The results, summarized in Figure 2, show that cells grown 48 hours in the presence of toxin are as susceptible to the second dose of toxin as those grown 48 hours without toxin. It is, therefore, apparent

FIGURE 1.

THE EFFECT OF ENTEROTOXIN B WHEN ADDED
AT THE TIME OF PLANTING OF HUMAN INTESTINE CELL CULTURES

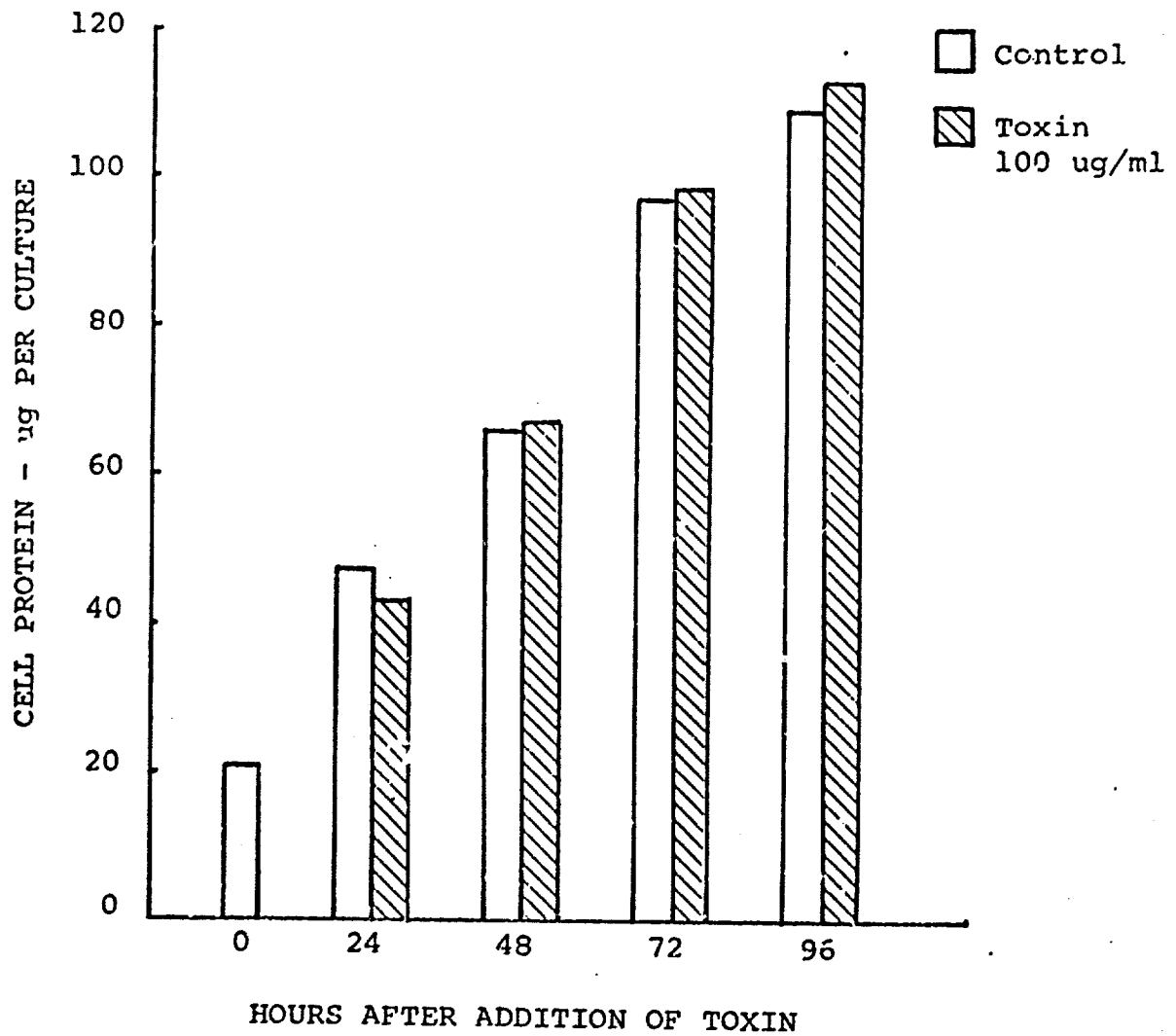
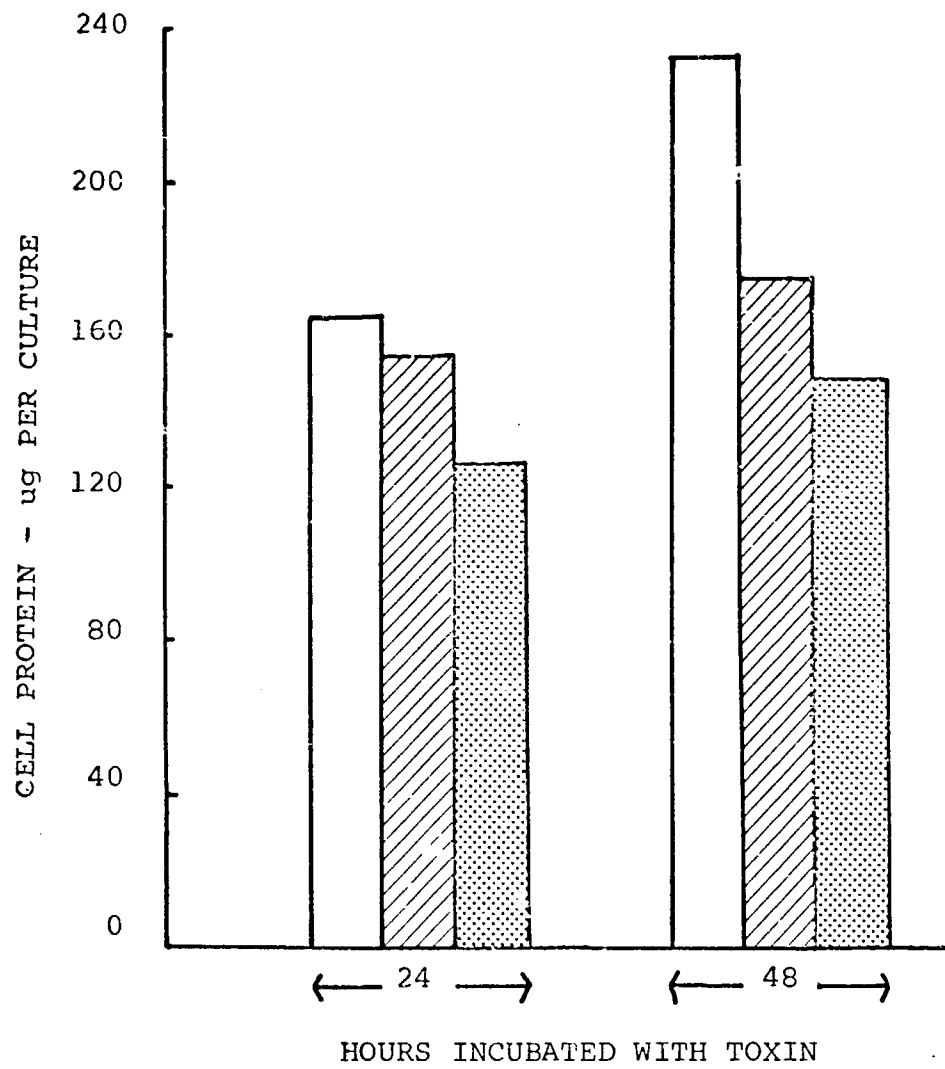





FIGURE 2.

GROWTH OF HUMAN INTESTINE CELLS
CHALLENGED WITH 100 $\mu\text{g}/\text{ml}$ OF ENTEROTOXIN B AT PLANTING
AND AFTER 48 HOURS OF GROWTH



-  Control
-  Toxin added once, at 48 hours
-  Toxin added twice, at 0 and 48 hours

that no permanent resistance to the enterotoxin is engendered by growth of the cell cultures, after trypsinization, in the presence of the enterotoxin.

TABLE 1. THE EFFECT OF ENTEROTOXIN B (100 µg/ml) ON HUMAN EMBRYONIC INTESTINE CELLS EXPOSED TO TRYPSIN

Time of Exposure to Trypsin (in seconds)	Cell Growth (Cell Protein) (% of control)
0	50
60	56
120	81
200	86
260	97
Control	100

Note: The cytotoxicity was measured by morphological changes and by inhibition of cell growth evidenced by reduction of cell protein per culture. The results are based on the 48 hour test.

Versene and Scraping. Furthermore, we investigated whether the enzymatic action of trypsin per se or the physical removal of the cells from the growing surface induces the temporary resistance of the cells to toxin. For comparison with the action of trypsin, Versene, a chelating agent, and scraping, as a non-chemical method, were used to suspend the cell monolayers.

To examine the effect of scraping, the medium was decanted from two-day-old culture tubes, and 1.0 ml of fresh growth medium was added. The cells were removed from the glass with a "rubber policeman" and were then suspended in medium by trituration. Immediately, 0.1 ml of toxin (100 µg) was added to each culture tube. Controls for scraped, trypsinized and versenized cells were incubated without toxin. Additional toxin controls consisted of two-day-old tube cultures to which 100 µg/ml toxin was added.

Table 2 summarizes the results based on morphological cytotoxicity. The results indicate that the scraped and the Versene-detached cells are as sensitive to enterotoxin B as are the two-day-old cell cultures, whereas those trypsinized are not sensitive.

From the data obtained in these experiments some inferences may be made regarding the acquired temporary resistance of human embryonic intestine cells to enterotoxin B.

TABLE 2. SUSCEPTIBILITY OF HUMAN EMBRYONIC INTESTINE CELLS TO ENTEROTOXIN B AFTER VARIOUS PLANTING PROCEDURES

Cell Cultures	Toxin (100 µg/ml)	Cytotoxicity*	
		(24 hours)	(48 hours)
Control	-	0	0
Control	+	2+	3+
Scraped	-	±	±
Scraped	+	3+	4+
Versenized	-	0	±
Versenized	+	3+	4+
Trypsinized	-	0	0
Trypsinized	+	0	0

*Scoring for Cytotoxicity

- 0 = no destruction of cell monolayer
- ± = less than 10% destruction
- 1+ = 10-25% destruction
- 2+ = 25-50% destruction
- 3+ = 50-75% destruction
- 4+ = 75-100% destruction

Since the removal per se of the cells from the glass growing surface appears not to be responsible for the acquired temporary resistance to enterotoxin B, we postulate that incubation with trypsin destroys some specific surface configuration which is required for the interaction of the cell with the toxin. Regeneration of the receptor sites appears to be effected within 48 hours after the enzymatic trypsin treatment.

In an attempt to explain the data showing resistance of cell cultures after trypsinization, a number of possibilities were considered. The first was that pinocytosis, the mechanism ascribed for the assimilation of larger molecular weight substances, might be adversely affected by trypsinization. However, the evidence (3) appears to favor an increased surface activity of recently trypsinized cells for pinocytosis.

The possibility of residual tryptic activity remaining with recently trypsinized cells is eliminated in the data presented (Table 1) which showed that cells trypsinized to the extent of being removed from the growing surface still retained some susceptibility to the enterotoxin. It was not until the cells which were all exposed to the same quantity of trypsin initially were allowed to remain in contact with the trypsin for extended time periods that full resistance

was obtained. Moreover, toxin was never added to the cell cultures until complete medium containing serum had first been added, thus inactivating any remaining trypsin.

The remaining possibility is that some specific surface configuration of the cell which is required for the interaction of the cell with the toxin is destroyed during the incubation with trypsin.

An analogous situation was recently reported by Zajac and Crowell (4,5). They showed that the incubation of HeLa cells with chymotrypsin inhibited the attachment of coxsackievirus B₃ and that incubation of HeLa cells with trypsin inhibited the attachment of poliovirus T₁. In addition, the investigators demonstrated that the concentration of trypsin for a given quantity of cells is critical for the inactivation to take place. They also showed that the regeneration of the receptor sites for both viruses was effected within 24-48 hours after the enzyme treatment.

Further experimentation utilizing fluorescent antibody techniques as well as radioactively-labeled enterotoxin is contemplated.

A manuscript based on this study has been prepared for publication: Schaeffer, W.I., J. Gabliks and R. Calitis.
Interference Caused by Trypsin upon the Interaction of Staphylococcal Enterotoxin B with Cell Cultures of Human Embryonic Intestine

2. Diphtheria Toxin

Work was curtailed during the second part of the year because of unavailability of personnel and labeled toxin. Previous studies have been published: Janis Gabliks and Marcia Falconer. "Diphtheria Toxin Interaction with Susceptible and Resistant Cell Cultures." Journal of Experimental Medicine, 123:723-732, 1966.

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II. SELECTED TOXIC SUBSTANCES

1. Insecticidal Compounds versus Virus Infections in Cell Cultures

The studies described in the Annual Progress Report of 1965 indicated that human HeLa cells exposed to subtoxic concentrations of the insecticidal compounds, Cygon^R, Dipterex^P, DI-Syston^R, chlordane and Karathane^R for 84 days were more susceptible to poliovirus infection than the corresponding control cells (1,2).

As it is recognized that residues of some agricultural insecticides persist in the body and that cells are continuously exposed to their metabolites, we investigated the possibility that these chemicals may alter certain physiological activities of cells and subsequently influence the susceptibility of hosts to virus infections.

The effects of organophosphorus, organochlorine and dinitrophenol insecticidal compounds on the replication of polio and of vaccinia viruses were studied in human Chang liver cells, using purified or analytical grade compounds obtained from their manufacturers. The organochlorine compounds were

- a) 1,1,1-Trichloro-2,2-bis(p-chlorophenyl)ethane, (DDT), technical p,p'-isomer 77.2%
- b) 1,2,4,5,6,7,8,8,-Octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene(chlordane), reference grade
- c) 4,4'-Dichloro- α -(trichloromethyl)benzhydrol (Kelthane), purity 96%

The organophosphorus compounds were

- a) O,O-Dimethyl(1,hydroxy-2,2,2-trichloroethyl)phosphonate (Dipterex), purity 100%
- b) O,O-Dimethyl S-(1,2-dicarbethoxyethyl)phosphorodithioate (malathion), purity 99.6%.

The dinitrophenol compound was

2-(1-Methylheptyl)-4,6-dinitrophenylcrotonate (Karathane), purity 88.4%.

The compounds were incorporated in Eagle's medium (3) (supplemented with 10% heat inactivated serum) 6 or 24 hours prior to the virus infection. The magnitude of virus replication was determined by titration of viral progeny from cultures harvested 24,48, and 72 hours after infection.

Parallel to the virus experiments, sets of identical tube cultures were incubated without virus to study the effects of insecticides on cell growth during the same incubation periods. The cytotoxicity was evaluated by changes in cell morphology and by growth inhibition, as has been described by Gabliks and Friedman (4).

When the compounds were tested in human Chang liver cultures below their acute toxicity levels (at approximately one-third of their growth inhibitory 50% levels), there was no morphological cytotoxicity detectable, but the growth was slightly reduced. The cell protein values were 94-85% of

the control values. Under the same experimental conditions the replication of vaccinia virus was markedly inhibited. The total yield of vaccinia progeny in the DDT, chlordane, Kelthane, Dipterex and malathion cultures was only 5-20% of the controls, and in the Karathane cultures, 63%.

In the poliovirus test the cell response was not uniform. In comparison to the controls, the virus yield in the DDT-treated cultures was slightly increased; the yield in the chlordane and malathion cultures was reduced (32 and 18% of the controls); and the yield in the Kelthane and Karathane cultures was greatly increased (430 and 1800% respectively).

When the yield of infectious virus per culture is expressed as yield per μ g of cell protein or per individual cell present in the insecticide-treated culture, the same relative magnitude of virus replication is evident. Accordingly, the reduction of virus yield appears not to be due to the slightly lower number of cells present in the insecticide-treated cultures, nor due to a direct inactivation of free virus by insecticides in medium, as was shown with the negative results of the virucidal contact tests.

In terms of the current research concerning the effects of insecticides, the biological significance and application of the results presented here can be considered from several aspects.

Since vaccinia, a DNA virus, was inhibited by all compounds tested (DDT, chlordane, Kelthane, Dipterex, malathion and Karathane), and polio, an RNA virus, was also inhibited by chlordane and malathion, some of the tested compounds warrant further investigation for antiviral activity. The stimulatory effect by DDT, Kelthane, and Karathane on poliovirus replication suggests a possibility that these compounds may have some specific effects on the mechanisms of viral biosynthesis. This possibility is supported by the results of our previous report which indicated an increased yield of poliovirus in HeLa cells exposed to Karathane for 77 days(2).

If the altered reactivity of cells to viruses is considered from the aspect of toxicology, the virus replication test may be useful in some cases as an indicator system for detection of alterations in cell metabolism induced by subtoxic concentrations of insecticides or other chemicals.

This study has been accepted for publication in the Archives of Environmental Health. The manuscript is attached as Appendix No. 1

2. Dipterex versus Vaccinia Virus Infection in Rabbits

The experiments conducted with organophosphorus compounds versus vaccinia infection in cell cultures demonstrated an inhibitory effect of Dipterex and malathion upon the replication of virus (see above). In order to determine the effects of these compounds (which are known as potent inhibitors of acetylcholinesterase) on virus infection in animals, we tested Dipterex in the rabbit.

The rabbits. Albino strain females weighing 2-3 kg were

maintained on Purina rabbit chow diet and water ad libitum.

In the first test the rabbits were injected intraperitoneally daily with Dipterex solution at 20 mg and 40 mg per kg of body weight for 8 days. The control animals received the same amount of physiological salt solution. Each group was composed of 3 to 4 rabbits.

On the third day after the first treatment, the animals were infected with vaccinia virus strain 971 H by infection intradermally of 0.1 ml of three different virus dilutions (10^{-2} , 10^{-3} , 10^{-4}). Each virus dilution was injected into two separate areas on the back of each rabbit.

During the course of the experiment, the weights, temperatures and skin lesions were recorded. The results are summarized in Table 3.

The average weights of the animals expressed as gains or losses from the original weights show no marked differences. The Dipterex-treated animals maintained or lost a small amount of their weight, whereas the control animals consistently gained weight. The loss of weight in the Dipterex-treated rabbits may be due partly to the slight toxicity of the compound and partly to the effects of the virus infection.

The average rectal temperature in the control rabbits increased up to 1.1°F during the acute stage of the infection while it increased only 0.6°F in the Dipterex-treated animals.

The vaccinia virus-induced skin lesions were classified arbitrarily as 1+ to 3+ exanthemas according to their size and intensity. The scores of lesions show that the exanthemas in the Dipterex-treated rabbits were less pronounced than in the control animals.

In order to assess the immune responses of rabbits to vaccinia infection, a virus neutralization test was performed using serum harvested 14 days after infection. The titer of neutralizing antibodies was determined by mixing 2 ml of each serum dilution in Eagle's medium and 2 ml of virus dilution containing 100 TCID₅₀'s. The antiserum-virus mixtures were incubated at 37°C for one hour, and then 1 ml of the mixture was added to Chang liver tube cultures. The average serum neutralization titer of the control group was dilution 1/170, whereas in the 40 mg/kg group it was 1/64, and in the 20 mg/kg, about 1/48 - about a three-fold titer difference.

The reduced titer of antibody in the Dipterex-treated rabbits may be explained by an inhibitory effect of Dipterex on the antibody producing system or by the reduced amount of viral antigens produced during the first infection which was relatively mild.

The protective action of antibody in vivo against reinfection with vaccinia virus was estimated by infecting the previously infected and some non-infected rabbits with virus dilutions containing 10 times more virus than that which was used during the first infection. Both the previously infected control rabbits and the Dipterex-treated rabbits showed marked resistance as evidenced by less intense skin

TABLE 3. EFFECTS OF DIPTEREX^R - ORGANOPHOSPHORUS INSECTICIDE ON VACCINIA VIRUS INFECTION
IN RABBITS

Parameter	Observation Period - Days from Infection																				
	Control				Dipterex -16 mg/kg				Dipterex - 8 mg/kg												
	-1	0	+1	+2	+3	+4	+5	-1	0	+1	+2	+3	+4	+5							
Weight change (g)	+4	-2	+26	+77	+88	+66	+188	-21	-33	-49	-12	-51	+7	+34	-52	-2	+5	-80	-55	+26	-46
Temperature (°F)	102.6	103.7	103.7	101.6	102.5	103.1	102.1	103.1	102.2	102.1	102.8	103.0	202.6	103.1	101.9						
Skin lesions (arb. scores)	+	1.5	2.5	1.8	±	0.6	0.6	0.6	0.6	0.6	0.6	0	0.8	0.6	0.6						

exanthemas than in the normal rabbits not previously infected with vaccinia virus.

In the second experiment the concentrations of Dipterex were increased up to 40 mg/kg of body weight. The rabbits were injected every day for 7 days, starting 2 days prior to the infection.

During the course of the infection the animals continued to gain weight. The results, based on changes in body temperatures and on the scores of vaccinia virus lesions, were not conclusive but, in general, did indicate an opposite effect, that is, a more severe disease in the rabbits treated with the highest concentrations of Dipterex.

In the third experiment the rabbits were injected twice daily since it is known that Dipterex is very rapidly metabolized and that its inhibitory effect on the activity of acetylcholinesterase lasts only about 8 hours (5).

Some of the rabbits which received 40 mg of Dipterex per kg of body weight twice daily died immediately after the second infection, whereas the treatment at the 20 mg/kg level was well tolerated for 6 days. The magnitude of infection in the Dipterex-treated animals was not markedly different from the controls, but the scores of vaccinia virus lesions indicated a more severe disease in all the treated groups and particularly in the prophylactic-therapeutic test group as compared to the therapeutic test group.

3. Dipterex versus Vaccinia Virus Infection in Mice

Albino female mice, weighing 12-13 g were injected intraperitoneally with various concentrations of Dipterex at the following intervals from the time of infection; -17, -2, +24, +48, +72, and +96 hours. For each concentration level (150 mg, 75 mg, 15 mg, and 7.5 mg/ kg of body weight) we used 15 mice, of which 10 were infected with vaccinia virus and 5 served as the toxicity controls for Dipterex. Vaccinia virus was diluted in Hanks' balanced salt solution, and 0.03 ml of a dilution 10^{-5} (approximately 100 TCID₅₀ levels) was injected into the mice intracerebrally.

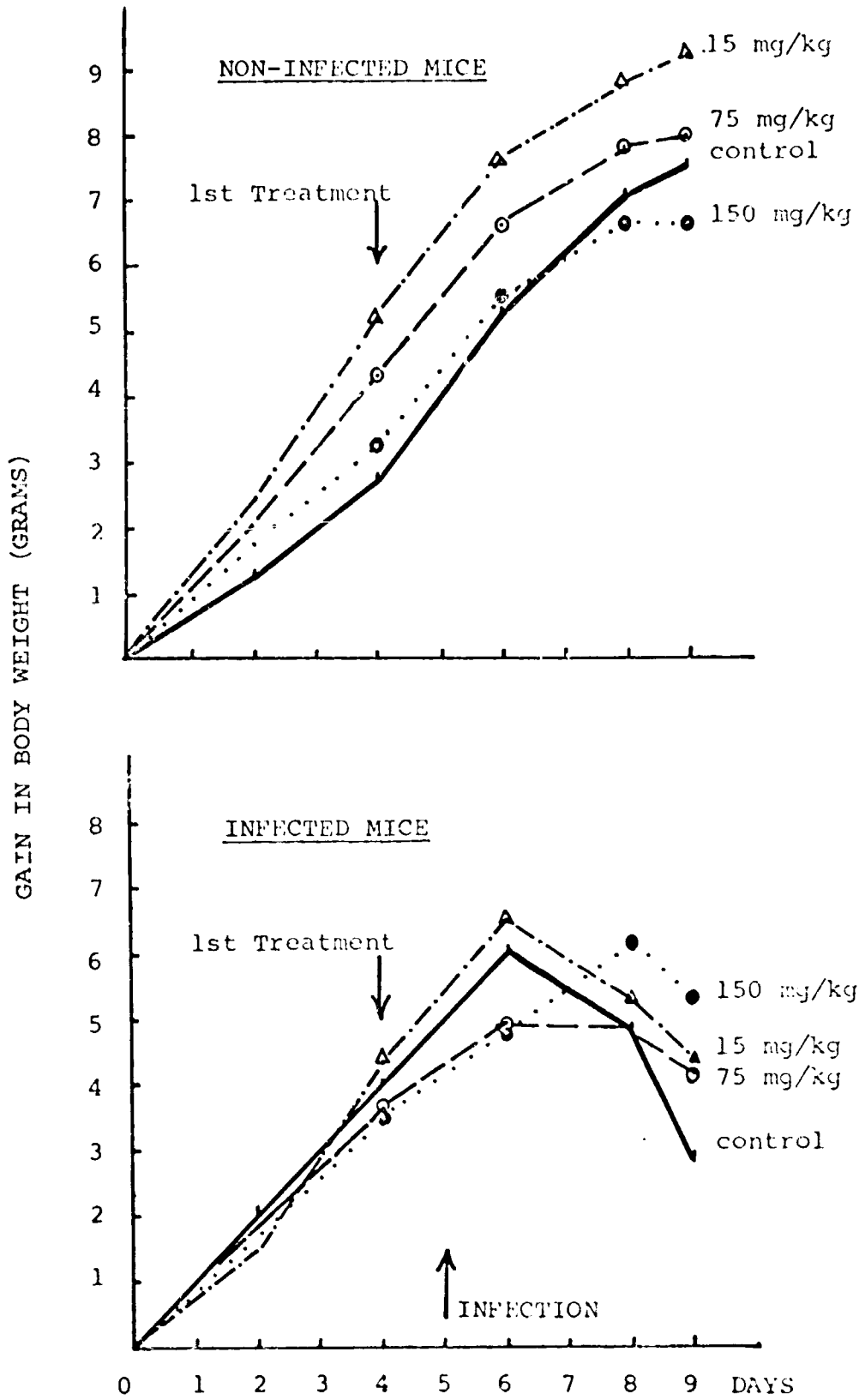
During the course of the treatment the mice did not show any detectable toxicity and continued to gain weight at a relatively constant rate as illustrated in Figure 3.

The infected mice exhibited the first signs of disease on the third day after infection, and at the same time they began to lose weight. Figure 3 shows the changes in weight. All control mice as well as the mice treated with 7.5 mg/kg of Dipterex died, whereas some of the animals in the highest dosage groups survived. At the 15 mg level 10% survived; at 75 mg, 20%; and at 150 mg, 30%.

To confirm the observed inhibitory effect of Dipterex on vaccinia virus infection, the second experiment was conducted using the same concentrations and the treatment schedules as described above. Additional groups of mice which were treated twice daily at 7 hour intervals were included as well.

FIGURE 3

EFFECT OF DIPTEREX^R - ORGANOPHOSPHORUS INSECTICIDE ON VACCINIA VIRUS REPLICATION IN MICE



In this test all the infected animals died. The average day of death in the treated groups was not significantly different from that in the control group.

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III. NUTRITIONAL FACTORS IN VIRAL INFECTIONS

1. Methionine and Glycine versus Vaccinia Infection

Our studies on the interrelationship of the nutritional state of hosts and virus diseases are concerned with the effects of a single amino acid deficiency on virus replication.

One of our first experimental objectives was the development of a model system in cell cultures for simultaneous evaluation of the effects of essential nutrients on cell growth and on virus replication. The importance of this aspect is evident, since a deficiency of an essential metabolite or any interference with its utilization leads to inhibition of cell growth, and the virus yield subsequently may be reduced by the reduction of the population of virus-susceptible cells. Consequently, it is difficult to determine the specific effects of nutritional deficiencies on viral biosynthesis.

This critical question was examined in our previous studies using the analogues of methionine (an essential amino acid) and glycine (a non-essential amino acid) in vaccinia virus-infected human Chang liver cultures. The magnitude of viral biosynthesis was evaluated by estimating the virus yield per individual cell or per μg of cell protein present in nutritionally deficient cultures and in the controls.

The data obtained by this method show that analogues of both methionine (l-ethionine) and of glycine (glycine methyl ester) suppressed vaccinia virus replication in human liver cells significantly more than they inhibited the growth of cells. The yield of vaccinia virus was also reduced when an excess of methionine and an excess of glycine was incorporated in the medium. This observation that a deficiency as well as an excess of both amino acids inhibited virus replication suggests the possibility that the balance of the intracellular amino acid pool may be as important for the proper replication of virus as it is for the maintenance and growth of higher organisms.

The inhibitory effect of l-ethionine on vaccinia virus replication was also established in rabbits injected with l-ethionine prophylactically. The skin lesions in the ethionine-treated rabbits were less pronounced and the body temperature lower than in the normal rabbits. Since the results obtained with ethionine in cell cultures and in the rabbit correlated well, the cell culture testing system appears to be a useful tool for comparing the effects of nutritional deficiencies or excesses on growth of cells and on virus replication.

The cell culture methods and the results are presented in the manuscript-appendix No. 2:

Gablík, J., Strachan, G., and Schaeffer, W.: Effect of Specific Amino Acid Deficiencies on Virus Replication in Cell Cultures. Proc. of the VII International Congress of Nutrition, Hamburg, Germany, 1966.

2. Leucine, Isoleucine and Valine versus Vaccinia Infection

In view of the fact that the balance of leucine, isoleucine and valine in diets affects the nutritional state of animals, it appeared tenable to investigate the effects of these amino acids on cell growth and on the replication of vaccinia virus.

The cell cultures, virus and test procedures were the same as described in our study with methionine and glycine (see Appendix No. 2).

A. Deficiency of leucine. To deplete the intracellular amino acid pool we used the method of Morton *et al.* (1). Cell monolayers of two-day-old tube cultures were exposed to 3 ml of Hanks' balanced salt solution at pH 7.5 for 6 hours at 37°C. The magnitude of amino acid depletion was not investigated.

Prior to the virus infection, groups of three cultures received Eagle's basal medium (2) without leucine or with leucine at concentrations approximately 1/3, 1/2 and 2/3 of the leucine concentration present in the normal Eagle's medium. In all tests the media contained 10% calf serum which had been dialyzed to remove the free amino acids and then heated at 56°C for 60 minutes to inactivate nonspecific viral inhibitors. Vaccinia virus (strain 971H) was diluted in the corresponding media, and each culture received approximately 100 TCID₅₀ levels according to the tube titration test.

Parallel sets of non-infected cultures served as the controls for cell growth. The growth was determined by counting the number of cells and by measuring the amount of cell protein per culture.

Table 4 shows the magnitudes of growth at the end of a 48 hour period. The increases in cell number and protein are proportional to the amount of leucine present in the medium. In normal Eagle's medium, which contains 26 µg of l-leucine per ml, the number of cells increased 182%, whereas in the leucine-free medium the increase was only 47% and the cells exhibited slight morphological cytopathogenicity.

The virus-induced cytopathic effect was scored using arbitrary units (1+ to 4+) based on the ratio of normal cells to virus-destroyed or affected cells; this effect is also indicated in Table 4. In the cultures without leucine the cytopathic effect was negligible. Moreover, it was also reduced in the cultures containing only 8.7 µg/ml of leucine. The results indicate a slower degeneration of leucine-deficient cells.

Table 5 summarizes the results on virus replication. The infectivity titers of viral progeny harvested from the leucine deficient cultures were lower than in the control and proportional to the amount of leucine present in Eagle's medium. If the total yield of infectious virus is expressed as the percentage of the control, the amount of virus in the leucine-free cultures was only 2% of the control.

As the virus yield is also partly dependent on the number of cells present in the cultures, the magnitude of viral

TABLE 4. THE EFFECT OF LEUCINE DEFICIENCY ON GROWTH OF HUMAN CHANG LIVER CELLS AND ON VACCINIA VIRUS INFECTION^a

Eagle's Medium l-leucine	Growth of Culture				Vaccinia Cytopathic Effect ^c
	Number of cells		Cell Protein		
(μ g/ml)	($\times 10^4$ /cult)	%Contr.	(μ g/cult)	%Contr.	
Contr. "0"hrs (b) 26.0	16.6 \pm 0.3 SD		128 \pm 6 SD		
Contr. (b) 26.0	47.6 \pm 0.5	100	258 \pm 7	100	2+
17.3	43.0 \pm 0.3	90.3	251 \pm 2	97	2+
13.0	41.5 \pm 0.6	87.8	251 \pm 10	97	2+
8.7	37.6 \pm 0.5	78.2	231 \pm 10	90	1+
0.0	24.4 \pm 0.2	51.2	138 \pm 11	54	\pm

^a48 hour test

^bComplete Eagle's Basal Medium (ref. 2)

^cDestruction of cell monolayer: \pm , 10%; 1+, 25%; 2+, 50%; 3+, 75%; 4+, 100%.

TABLE 5. REPLICATION OF VACCINIA VIRUS IN HUMAN CHANG LIVER CELLS IN LEUCINE-DEFICIENT MEDIUM^a

Eagle's Medium l-leucine	Virus Titer-TCID ₅₀	Virus Yield (TCID ₅₀) - Per Cent of Control		
		Per Culture	Per Cell	Per μ g Cell Protein
(μ g/ml)	(Log ₁₀)	(%)	(%)	(%)
Contr. (b) 26.0	5.3	100	100	100
17.3	5.0	47	48	52
13.0	4.6	21	22	24
8.7	4.6	21	24	27
0.0	3.6	2	4	4

^a48 hour test

^bComplete Eagle's Basal Medium (ref.2)

biosynthesis should be evaluated by comparing the virus yield (TCID₅₀'s) per individual cell and per μg of cell protein in the nutritionally deficient cultures. When the results are expressed as percentages of the control, as shown in Table 5, the yields per cell are also markedly reduced, and the magnitude of reduction is proportional to the amount of leucine present in the medium. Therefore, the reduction in virus yield is primarily an effect of leucine requirement for vaccinia biosynthesis and is not due to the reduced number of cells present in the leucine-deficient cultures.

The inhibitory effect of leucine deficiency on cell growth and on vaccinia virus replication is compared in Figure 4. In general, the virus yield was relatively more inhibited than was the growth of cells. For example, at 13 $\mu\text{g}/\text{ml}$ of leucine, the growth was 80% of the control but the virus yield only 22% of the control.

B. Excess of leucine, isoleucine and valine. Eagle's basal medium (2), which contains among other amino acids 26 μg l-leucine, 26 μg l-isoleucine, and 24 μg l-valine per milliliter, was supplemented with 10% dialyzed heat-inactivated calf serum and served as the control medium.

When the medium was supplemented with an additional 930 μg l-leucine, 930 μg l-isoleucine and 830 μg l-valine individually or with all three amino acids simultaneously, the growth of Chang liver cells was inhibited. Table 6 shows the growth of cultures and the differences in cell number and protein as percentages of the controls.

In all cases the growth-inhibitory effect was small during the first 24 hours of incubation but progressed with increased time. In the cultures which contained the increased amounts of all three amino acids, the growth ceased after 24 hours, and the cells also exhibited slight changes in morphology.

Parallel sets of cultures, infected with 5020 TCID₅₀ levels of vaccinia virus showed the characteristic cytopathic effect at 48 hours. In all cultures containing the increased amounts of these amino acids, the degenerative changes were less pronounced than in the controls.

The results on viral biosynthesis are summarized in Table 7. The infectivity titers of viral progeny were reduced by an excess of leucine, isoleucine and valine when each was tested alone and by the combination of all three amino acids. The inhibitory effect on viral biosynthesis is also evident when the virus yields per individual cell or per μg of cell protein are compared to the corresponding controls.

If the inhibitory effects using an excess of leucine, isoleucine and valine on the cell growth and on the replication of virus are compared, as illustrated in Figure 5, it is evident that the replication of virus was more suppressed than the growth of cells. The significance of these findings, however, should be considered in view of the fact that the

TABLE 6. THE EFFECT OF AN EXCESS OF LEUCINE, ISOLEUCINE AND VALINE ON GROWTH OF HUMAN CHANG LIVER CELLS AND ON VACCINIA VIRUS INFECTION^a

Eagle's Medium	Concentr. ($\mu\text{g/ml}$)	Growth of Culture		Vaccinia Cytopathic Effect ^c		
		Number of Cells ($\times 10^4/\text{cult}$) %Contr.	Cell Protein ($\mu\text{g/cult}$) %Contr.			
Contr. "0" hrs (b)		37.3 \pm 0.9 SD	218 \pm 12 SD			
Contr. (b)		94.3 \pm 5.4	100	414 \pm 2	100	4+
l-Leucine	930	80.5 \pm 6.0	85	374 \pm 2	90	3+
l-Iso- leucine	930	80.3 \pm 6.0	85	350 \pm 5	85	3+
l-Valine	830	83.1 \pm 2.4	88	385 \pm 13	93	3+
l-Leucine	930	57.2 \pm 1.8	61	309 \pm 8	75	2+
l-Iso- leucine	930					
l-Valine	830					

^a48 hour test

^bComplete Eagle's Basal Medium (ref. 2) contains l-leucine, 26 $\mu\text{g/ml}$; l-isoleucine, 26 $\mu\text{g/ml}$; and valine, 24 $\mu\text{g/ml}$

^cDestruction of cell monolayer: \pm , 10%; 1+, 25%; 2+, 50%; 3+, 75%; 4+, 100%.

TABLE 7. REPLICATION OF VACCINIA VIRUS IN HUMAN CHANG LIVER CELLS IN MEDIUM CONTAINING AN EXCESS OF LEUCINE, ISOLEUCINE, AND VALINE^a

Eagle's Medium	Concentr. ($\mu\text{g/ml}$)	Virus Titer-TCID ₅₀ Log ₁₀	Virus Yield (TCID ₅₀) - % of Control		
			Per Culture (%)	Per Cell (%)	Per μg Cell Prot. (%)
Control (b)		5.7	100	100	100
l-Leucine	930	4.7		11	11
l-Isoleucine	930	4.7		11	12
l-Valine	830	5.2		36	34
l-Leucine	930	5.2		52	42
l-Isoleucine	930				
l-Valine	830				

^a48 hour test

Complete Eagle's Basal Medium (ref. 2) contains l-leucine, 26 $\mu\text{g/ml}$; l-isoleucine, 26 $\mu\text{g/ml}$; and valine, 24 $\mu\text{g/ml}$.

FIGURE 4

EFFECT OF LEUCINE CONCENTRATION
ON GROWTH OF CELLS AND VACCINIA VIRUS

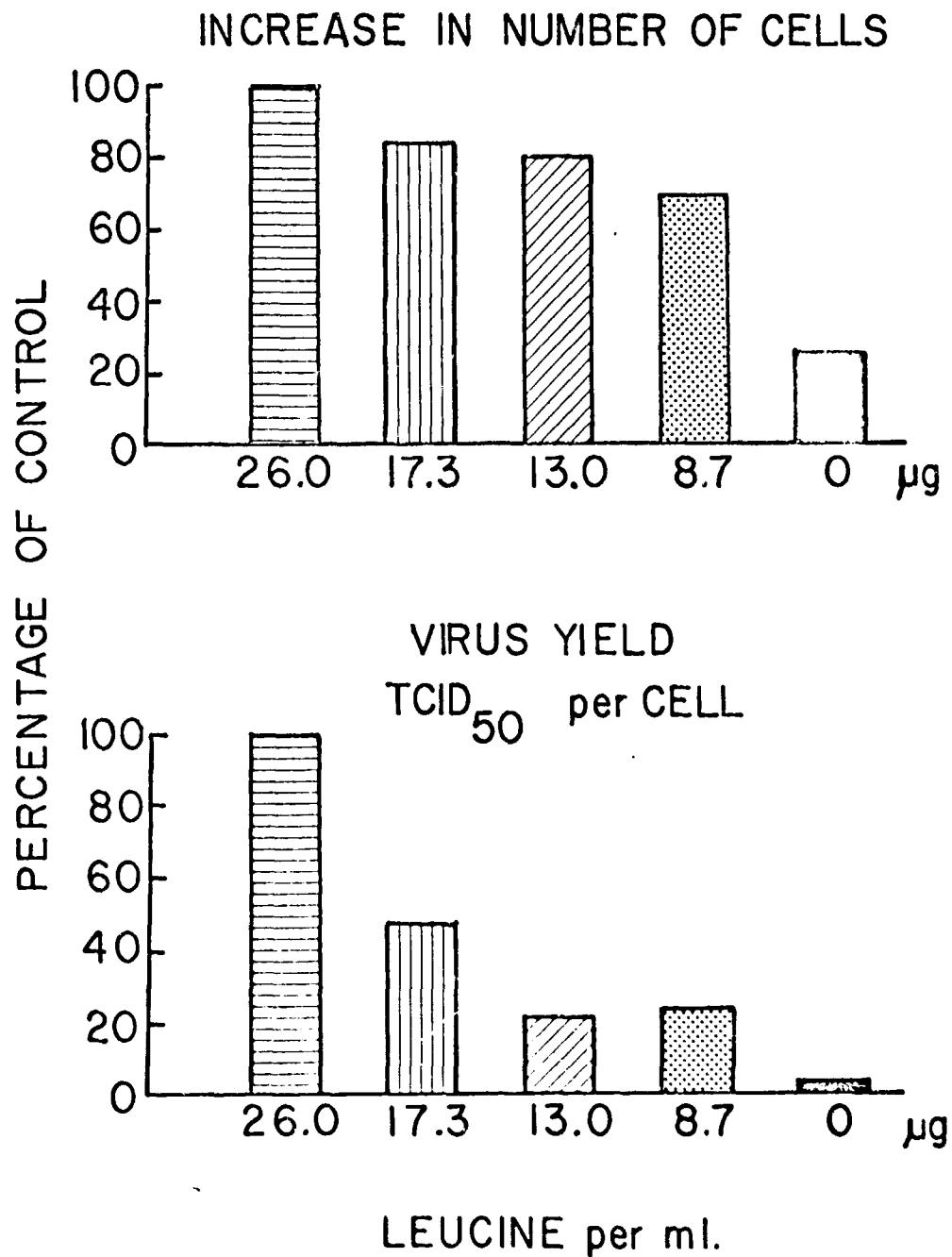
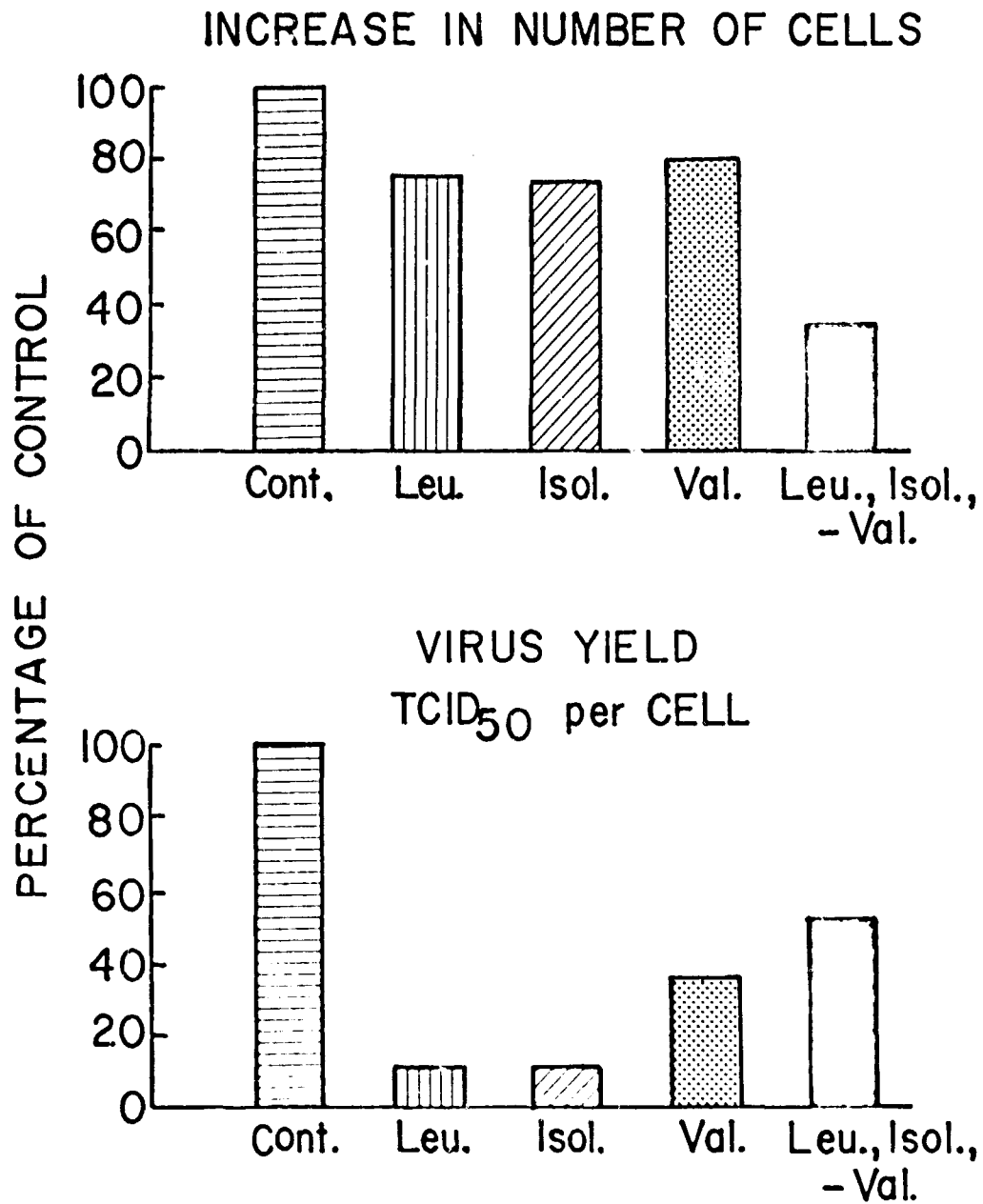


FIGURE 5

EFFECTS OF EXCESS OF AMINO ACIDS ON
GROWTH OF CELLS & VACCINIA VIRUS



concentrations of these three amino acids were increased to about 36 times the concentrations present in the Eagle's control medium. Accordingly, it appears that the use of an excess of a single amino acid is not very effective in altering the replication of virus.

From these data it is evident that either a deficiency of leucine or an excess of leucine, isoleucine and valine is inhibitory for vaccinia virus biosynthesis in human Chang liver cells. These findings suggest the possibility that the balance of amino acids in the intracellular amino acid pool may be important for proper virus replication.

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Appendix Number 1

EFFECTS OF INSECTICIDAL COMPOUNDS ON THE REPLICATION OF
VACCINIA AND POLIO VIRUSES IN HUMAN CHANG LIVER CELLS^{1, 2, 3}

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INTRODUCTION

It is recognized that residues of agricultural insecticides are present in the food supply and that a regular intake of small amounts of insecticides leads to the accumulation of some organochlorine compounds in animal and human tissues (1-5). For example, DDT, dieldrin, lindane, endrin, and heptachlor epoxide have been found in human fat and other tissues (4,5). As a result of insecticide persistence in the body, cells are continuously exposed to their metabolites. Therefore, one must consider the possibility that these chemicals may alter some physiological activities of cells, subsequently influencing the susceptibility of animals to other biologically active agents, including viruses.

Since virus replication is dependent upon the metabolism of host cells, we postulated that some alterations in cell metabolism may influence the mechanism of viral biosynthesis. This possibility is supported by our previous observations that chronic exposure of cultivated cells to insecticides alters their susceptibility to diphtheria toxin and to poliovirus infection (6-8).

To investigate the effects of insecticidal compounds on animal virus replication, we tested six insecticidal compounds in human liver cells infected with vaccinia and poliovirus.

MATERIALS AND METHODS

Cell culture. All cytotoxicity and virus infection tests were performed in heteroploid cell culture - human Chang liver(9). The stock culture was obtained from Microbiological Associates, Inc., Bethesda, Maryland and was maintained by serial passages in Eagle's basal medium (10) supplemented with 10% calf serum. In all tests the medium contained 100 units per ml penicillin and 100 μ g per ml streptomycin. For cytotoxicity tests, cells were harvested from stock cultures by subjecting them to the action of 0.25% trypsin solution. The cells were then re-suspended in growth medium at a concentration of 8×10^6 cells per ml, and 1 ml volumes were planted in screw-capped culture tubes. Cultures were incubated in a stationary position at 36 to 37°C for two or three days until a confluent cell-monolayer developed on the glass.

Insecticidal compounds. The insecticides investigated in the study were purified or analytic grade compounds obtained from their manufacturers. The organochlorine compounds were: 1,1,1-Trichloro-2,2-bis(p-chlorophenyl)ethane (DDT)¹, technical p,p'-isomer 77.2%; 1,2,4,5,6,7,8,8-Octachloro-2,3,3a,4,7,7a-hexahydro-4,7, methanoindene (chlordane)², reference grade; 4,4'-Dichloro- α -(trichloromethyl) benzhydrol (Kelthane^R)³, purity 96%. The organophosphorous compounds were: O,O-Dimethyl (1-hydroxy-2,2,2-trichloroethyl) phosphonate (Dipterex^R)⁴, purity 100%; O,O-Dimethyl S-(1,2-dicarbethoxyethyl) phosphorodithioate (malathion)⁵, purity 99.6%. The dinitro-phenol compound was 2-(1-Methylheptyl)-4,6-dinitrophenylcrotonate (Karathane^R)³, purity 88.4%.

For testing, the water soluble compound, Dipterex, was dissolved in the growth medium directly. All other compounds were water insoluble and were first dissolved in ethyl alcohol and then diluted in the medium by a 100-fold step. As determined in previous experiments, an ethyl alcohol concentration in the medium below 0.1% was not toxic to the cells. Insecticide stock solutions were adjusted to pH 7.5 with NaHCO₃ or NaOH solutions when necessary.

Test procedures. All cytotoxicity and virus infection tests were performed in cell-monolayer tube cultures. Insecticides at two different concentrations were incorporated in Eagle's medium (which contained 10% heat inactivated serum) and were tested in triplicate. In vaccinia virus experiments the insecticide-treated cultures were incubated for 6 hr and were then infected with 0.1 ml of vaccinia virus dilution containing 316 TCID₅₀ (tissue culture infective dose 50%). The vaccinia virus strain 971H (from Dr. John F. Enders, Children's Hospital, Boston, Mass.) was a pool harvested from infected chorioallantoic membranes of embryonated chicken eggs.

In the poliovirus experiments the cells were incubated in the presence of insecticides for 24 hr prior to the virus infection. At the time of infection the medium was removed and the cultures received 1 ml of fresh medium, containing the same concentration of insecticides and 1000 TCID₅₀ of poliovirus, Lansing (type 2)

strain adapted to human amnion cells (from Dr. John F. Enders, Children's Hospital, Boston, Mass.).

The virus-induced cytopathogenic effect was evaluated by microscopic examination of the cultures. The magnitude of virus replication was determined by harvesting the cultures 24, 48, and 72 hr after infection. The cultures were stored at -40°C , and, for titration of progeny, virus was liberated from the cells by repeated freezing and thawing. The virus yield was measured by titration of its infectivity in human Chang liver cells, using the tube titration method (11). The highest dilution of virus producing infection in 50% of the cultures was designated as the TCID_{50} . This value was calculated, by the method of Reed and Muench (12), from the cytopathogenicity results obtained from 4 to 5 cultures at each virus dilution.

To determine a possible virucidal effect of the insecticides on the free viruses in the culture medium, the following contact test was performed. Vaccinia and polio viruses, at concentrations 100 times their ordinary testing levels, were added to the culture medium containing insecticides at the highest test concentration used, and the virus-compound mixtures were incubated at 37°C for 3 hr. The same mixtures, stored at 4°C in an ice bath for the same length of time, served as controls. After the incubation period the virus-compound mixtures were diluted in medium and their infectivity titers determined as described above.

Cytotoxicity tests. Parallel to the virus experiments, sets of identical cultures were incubated without virus to study the effects of insecticides on cell growth during the same incubation periods. The cytotoxicity was evaluated by changes in cell morphology and by growth inhibition, as has been described by Gabliks and Friedman (6).

Based on previous results, the test concentrations of insecticides were selected from those levels which did not induce marked cytotoxicity. Three cultures were used for each concentration group in every test performed. These cultures were removed 24, 48, and 72 hr after incubation for microscopic examination, cell count, and cell protein determination.

Culture growth was expressed as increases of cell protein and cell number per culture, the increase of cell protein being proportional to the increase in the number of cells (13, 14). In order to measure protein, cell monolayers were washed twice with balanced salt solution to remove all traces of protein present in the test medium. The washed cells were then dissolved in Lowry's solution, and the protein content was determined with the Folin-Ciocalteu reagent according to the method of Oyama and Eagle (13).

The number of cells per culture was estimated by counting cell nuclei. The cells were suspended in a citric acid and crystal violet solution (11) to dissolve the cytoplasm, and then the strained nuclei were counted in a hemacytometer.

RESULTS

Vaccinia virus infection. The comparative effects of DDT on the growth of human liver cells and on vaccinia virus replication are summarized in Figure 1. In the presence of 30 μ g of DDT per ml of medium there was no cytotoxicity detectable by microscopic examination. However, the growth curves based on cell count indicated progressive inhibition of growth. When the number of cells in the cultures is expressed as percentages of their corresponding controls (as indicated in the lower portion of Figure 1), the inhibitory effect is relatively small during the early periods of incubation, but increases progressively with time. At 24 hr the number of cells in the DDT-treated cultures was 85% of those in the control; at 48 hr, 69%; and at 72 hr, 57%.

The progressive reduction of cell growth is also evident from the growth curves based on the cell protein values per culture. After incubation for 24 hr, the protein values were 92% of the control values, and at 48 hr, 85%.

The magnitude of virus replication is indicated on the right side of Figure 1. In the DDT-treated cultures the infectivity titer of progeny, TCID₅₀, was 0.8 to 1.0 logarithm lower than in the control. When the infectivity titer is expressed as total TCID₅₀'s per culture, the total virus yield in the DDT cultures at 24 hr is only 14% of the virus yield in the control cultures, and at 48 hr, 20%.

In order to evaluate the possibility that the reduction of virus yield is not due to the lower number of cells present in the DDT-treated cultures, we calculated the ratio of the TCID₅₀ to the cell number or to the amount of cell protein present in the DDT-treated cultures. Figure 2 shows that during the first 24 hr after infection the DDT-treated cell released only 18% of the virus released by the control cell. At 48 hr, the yield was increased about 5 times in the normal cell and about 4 times in the DDT-treated cell, but it was still only 13% of that in the normal cell. The same magnitude of virus replication is also evident when the virus yield is expressed per μ g of cell protein, as shown on the right side of Figure 2.

Using the same methods as outlined for DDT, we also tested chlordane, Kelthane, Dipterex, malathion, and Karathane. The results at 48 hr of incubation are summarized in Table 1. At the indicated concentrations of these insecticides, which were several times below their growth inhibitory ID₅₀ levels determined previously, and which did not induce any observable morphological cytotoxicity, the infectivity titers of progeny were reduced with all compounds. The virus yield per culture was reduced to 53% for Karathane and to 5% for Kelthane. Although the growth of insecticide-treated cultures was not measured in parallel sets of non-infected cultures, additional tests showed that, at the indicated test concentrations, none of the compounds reduced total cell protein by more than 10% of the control values.

When the compounds were tested for possible virucidal effect, the infectivity titers of vaccinia virus exposed to insecticides for 3 hr before infection did not differ from their corresponding controls.

Poliovirus infection. In the poliovirus experiments the

virus solution was added 24 hr after the addition of insecticides. In the presence of 20 and 40 μg levels of DDT, poliovirus yield per culture was comparable to that of the control. However, when the yield of infectious virus released is expressed per individual cell or per μg of cell protein, as shown in Figure 3, it is evident that at the 20 and 40 μg levels of DDT, the yield of virus per cell was increased 37 and 90%, respectively. Similarly, the yield per μg of protein was also increased 15 and 47%, respectively.

The effects of other insecticides on poliovirus replication are summarized in Table 2. At the indicated levels, the insecticides did not induce any detectable morphological cytotoxicity, but the total cell protein per culture was slightly reduced, ranging from 92 to 87% of that of the control. Under these conditions the total virus yield in the chlordane-treated culture was 32% of the control, and in the malathion-treated cells, 18% of the control. In contrast to the inhibitory action of these two insecticides, the Kelthane-treated cultures produced about four times more virus, and the Karathane-treated cells 18 times more virus than the corresponding controls. The same relative magnitude of virus yield is also evident when the yields are expressed per μg of cell protein present in those cultures. When the compounds were tested for possible virucidal effects on free virus, the infectivity titers of poliovirus incubated with insecticides before addition to cell cultures did not differ from their corresponding controls.

DISCUSSION

Insecticidal compounds DDT, chlordane, Kelthane, Dipterex, malathion, and Karathane have been previously studied in human Chang liver and HeLa cells, and their acute and chronic toxicity levels have been reported (6,7).

When the same compounds were tested in Chang liver cultures below their acute toxicity levels (at approximately one-third of their growth inhibitory 50% levels), there was no morphological cytotoxicity detectable, but the growth was slightly inhibited. The cell protein values were 94 to 85% of the control values. Under the same experimental conditions the replication of vaccinia virus was markedly inhibited. The total yield of vaccinia progeny in the DDT, chlordane, Kelthane, Dipterex and malathion cultures was only 5 to 20% of the controls, and in the Karathane culture, 63%

In the poliovirus tests the cell response was not uniform. In comparison to the controls the virus yield in the DDT-treated cultures was slightly increased, the yield in the chlordane and malathion cultures was reduced (32 and 18% of the controls), and the yield in the Kelthane and Karathane cultures was greatly increased (430 and 1800%, respectively).

When the yield of infectious virus per culture is expressed as yield per μg of cell protein or per individual cell present in the insecticide-treated culture, the same relative magnitude of virus replication is evident, except for the increased yield

of poliovirus per cell in the DDT-treated cultures. Accordingly, the reduction of virus yield appears not to be due to the slightly lower number of cells present in the insecticide-treated cultures, nor due to a direct inactivation of free virus by insecticides in medium, as was shown with the negative results of the virucidal contact tests.

In terms of the current research concerning the effects of insecticides, the biological significance and application of the results presented here can be considered from several aspects.

Since vaccinia, a DNA virus, was inhibited by all compounds tested (DDT, chlordane, Kelthane, Dipterex, malathion, and Karathane) and polio, an RNA virus, was also inhibited by chlordane and malathion, some of the tested compounds warrant further investigation for antiviral activity. The stimulatory effect by DDT, Kelthane, and Karathane on poliovirus replication suggests a possibility that these compounds may have some specific effects on the mechanisms of viral biosynthesis, and the state of some latent viruses may also be altered. This possibility is supported by the results of our previous report which indicated an increased yield of poliovirus in HeLa cells, chronically exposed to Karathane for 77 days (7,8).

In view of the possible effects of insecticides on the pathogenesis of animal viral diseases, one must consider the concentrations tolerated in tissues of various organs of an intact organism. Unfortunately, the present available data on the toxicity of insecticides in different animal species does not warrant any direct comparison of the reported LD₅₀ levels to the concentrations used in this study. It is recognized that the toxic effects in animals usually depends on adsorption, metabolism, storage and excretion of compounds; however, most of these functions are not present in tissue cultures which are isolated from the circulatory, nervous or endocrine controls. In order to evaluate this important question of tolerated tissue concentrations, we have initiated studies with tolerated levels of these compounds versus virus infections in experimental animals.

If the altered reactivity of cells to viruses in the presence of insecticides is considered from the aspect of toxicology, the virus replication test may be useful in some cases as an indicator system for detection of some alterations in cell metabolism induced by subtoxic concentrations of insecticides or other chemicals.

SUMMARY

Insecticidal compounds DDT, chlordane, Kelthane^R, Dipterex^R, malathion, and Karathane^R at subtoxic concentrations inhibited vaccinia virus replication in human Chang liver cells. Under the same experimental conditions, the replication of poliovirus was inhibited only by chlordane and malathion, whereas Kelthane and Karathane increased the virus yields 4 and 18 times, respectively, and DDT exhibited a light stimulatory effect.

Since the reduction in virus yields was not due to extracellular inactivation of virus, the results suggest that some insecticides exert an antiviral activity by altering some physiological activities of cells. Consequently, the magnitude of virus

replication may be useful as a parameter for the detection of toxicity in insecticides and other chemicals below their acute toxicity levels.

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TABLE I
EFFECT OF INSECTICIDES ON GROWTH OF HUMAN CHANG LIVER CELLS AND ON
VACCINIA VIRUS REPLICATION*

INSECTICIDAL COMPOUND	TEST CONC.	CELL		GROWTH		VIRUS YIELD (TCID ₅₀)/CULTURE		
		MORPHOL. TOXICITY OF COMPOUND		CELL PROTEIN PERCENT OF CONTROL		TITER DIFFER. FROM CONTROL	TCID ₅₀ /μg CULTURE PERCENT OF CONTROL	TCID ₅₀ /μg CELL PROT. PERCENT OF CONTROL
	μg/ml					Log ₁₀	%	%
<u>ORGANOCHLORINE</u>								
DDT	30.0	0		85		-0.7	20	10
CHLORDANE [®]	5.0	0		--		-0.8	16	--
KELTHANE [®]	1.0	0		--		-1.3	5	--
<u>ORGANOPHOSPHORUS</u>								
DIPTEREX [®]	10.0	0		--		-1.0	10	--
MALATHION	10.0	0		--		-0.8	20	--
<u>DINITROPHENOL</u>								
KARATHANE [®]	0.1	0		95		-0.2	63	94

*INCUBATED 48 HR

TABLE II
EFFECT OF INSECTICIDES ON GROWTH OF HUMAN CHANG LIVER CELLS AND ON
POLIOVIRUS REPLICATION*

INSECTICIDAL COMPOUND	CELL GROWTH			VIRUS YIELD (TCID ₅₀)/CULTURE		
	TEST CONC.	MORPHOL. TOXICITY OF COMPOUND	CELL PROTEIN PERCENT OF CONTROL	TITER DIFFER. FROM CONTROL	TCID ₅₀ /μg CULTURE PERCENT OF CONTROL	TCID ₅₀ /μg CELL PROT. PERCENT OF CONTROL
	μg/ml		%	Log ₁₀	%	%
<u>ORGANOCHLORINE</u>						
DDT	20.0	0	87	0.0	100	119
CHLORDANE	5.0	0	94	-0.51	32	35
KELTHANE [®]	1.0	0	89	+0.53	430	480
<u>ORGANOPHOSPHORUS</u>						
MALATHION	10.0	0	93	-0.75	18	19
<u>DINITROPHENOL</u>						
KARATHANE [®]	0.1	0	92	+1.25	1800	1900

* INCUBATED 48 HR

FIGURE LEGENDS

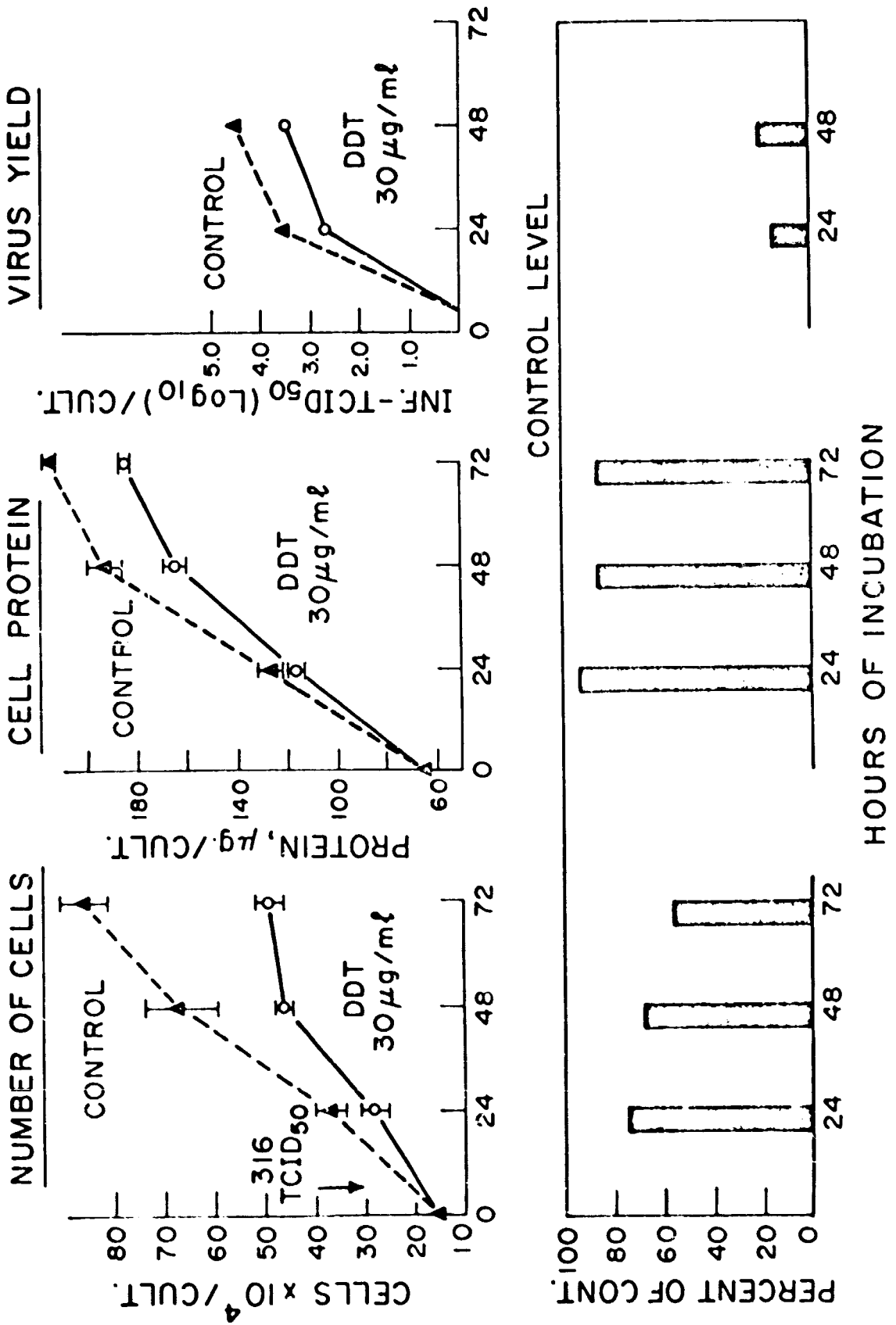
- Figure 1. Effect of DDT on the growth of human Chang liver cells and on vaccinia virus replication. Cells were exposed to 30 μ g of DDT for 6 hr and then infected with vaccinia virus. The differences in cell growth (measured by cell counts and cell protein determination) and in virus replication (measured as infectivity titers of progeny) are expressed as percentages of their controls on the lower part of the graph. The vertical lines on growth curves indicate the standard deviations of the results.
- Figure 2. Vaccinia virus replication in the DDT-treated human Chang liver cells, expressed as (TCID₅₀) yields of virus progeny per individual cell and per μ g of cell protein. The virus yield in the DDT-treated cell is reduced.
- Figure 3. Poliovirus replication in the DDT-treated human Chang liver cells, expressed as (TCID₅₀) yields of virus progeny per individual cell and per μ g of cell protein. The virus yield in the DDT-treated cell is increased.

FOOTNOTES

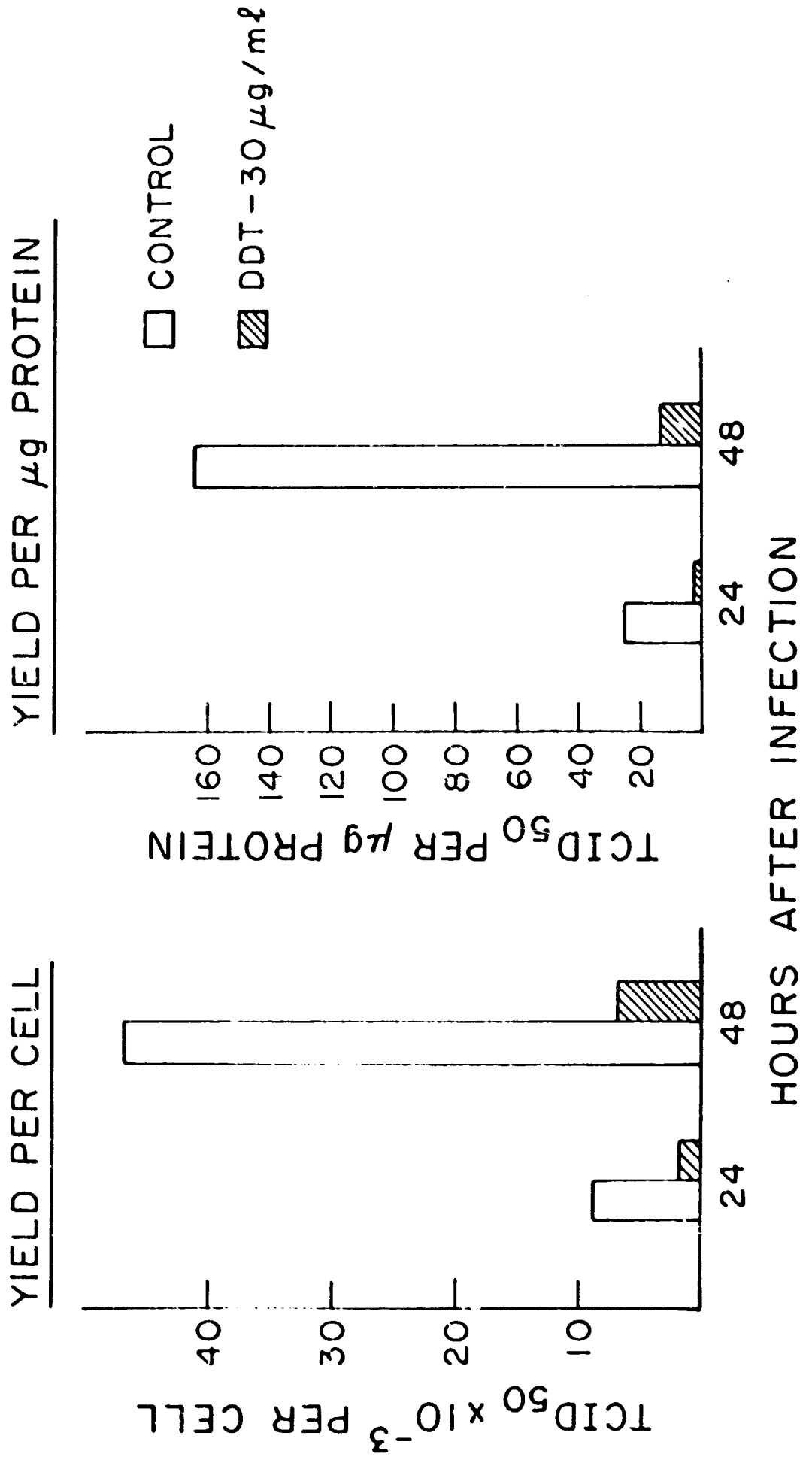
- ¹ Edcan Laboratories, South Norwalk, Connecticut.
- ² Velsicol Corp., Chicago, Illinois.
- ³ Rohn and Haas Company, Philadelphia, Pennsylvania.
- ⁴ Chemagro Corp., Kansas City, Missouri.
- ⁵ American Cyanimid Co., Princeton, New Jersey.

We are grateful to these companies for contributing the chemicals used in this study.

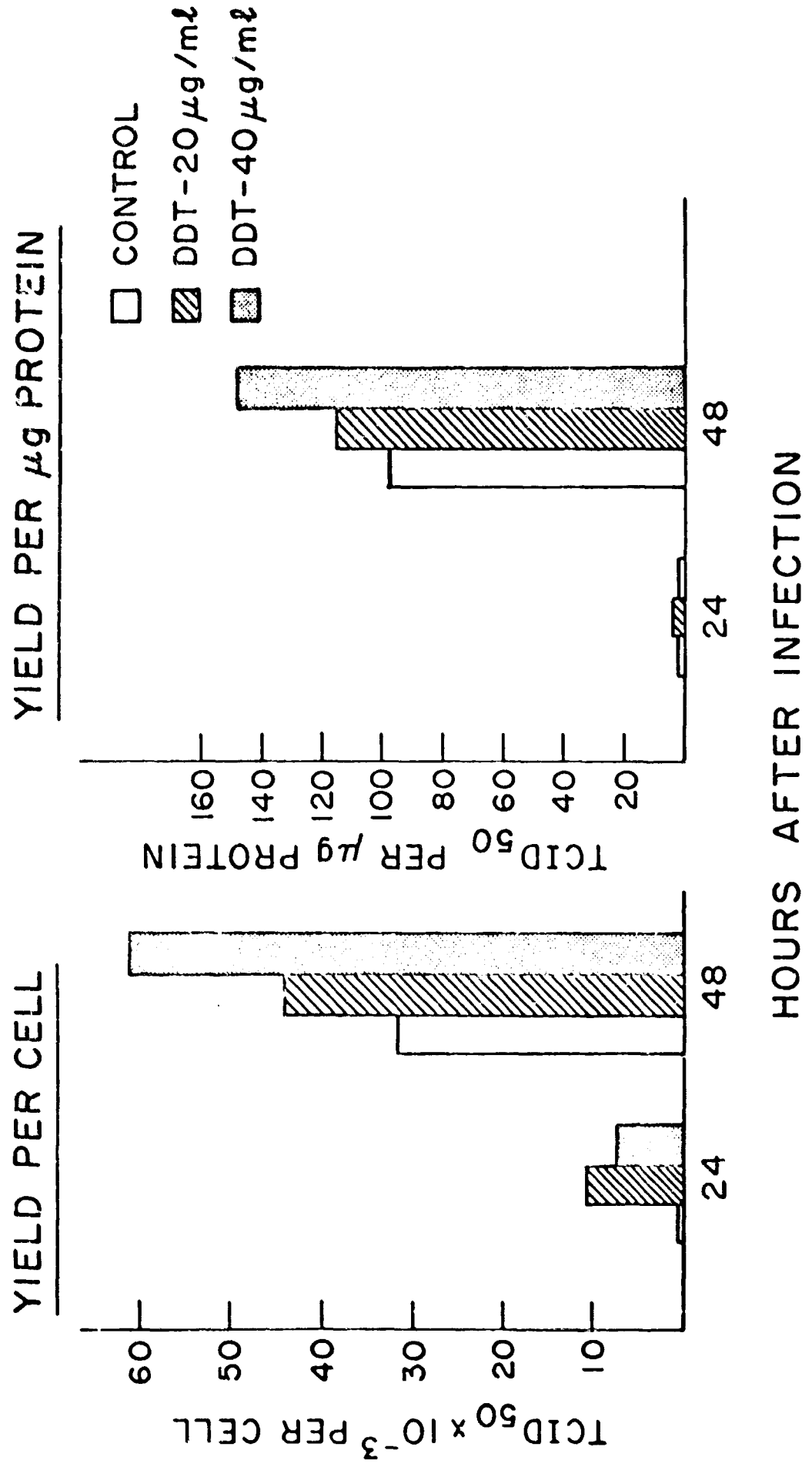
EFFECT OF DDT ON GROWTH OF HUMAN CHANG LIVER CELLS
AND ON VACCINIA VIRUS REPLICATION



VACCINIA VIRUS REPLICATION IN DDT TREATED
 HUMAN CHANG LIVER CELL CULTURES



POLIOVIRUS REPLICATION IN DDT TREATED
HUMAN CHANG LIVER CELL CULTURES



Appendix Number 2

EFFECT OF SPECIFIC AMINO ACID DEFICIENCIES ON VIRUS
REPLICATION IN CELL CULTURES ^{1,2}

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August 3-10, 1966

³with the technical assistance of Marcia Falconer and Ruta Calitis

An imposing number of publications on the interaction of nutrition and infection has been reviewed by Scrimshaw, Taylor and Gordon (1,3) and Scrimshaw (2). The authors classify the nutritional state of a host as synergistic with, or antagonistic to, infections. In general, a deficiency in nutritional factors acts synergistically with infections; however, some specific deficiencies of amino acids and vitamins exhibit an antagonistic effect, especially in the case of virus infections. Apparently, changes in the metabolism of cells affect the multiplication rate of intracellular parasites, as has been shown with foot and mouth disease, vaccinia, western equine encephalitis, avian encephalomyelitis, poliomyelitis, influenza and other viruses (3).

To evaluate the specific effect of a single nutrient on virus replication quantitatively, many investigators have used amino and nucleic acid analogues in tissue and cell cultures. When an analogue of a metabolite is introduced into a biological system,

It may reduce the effective concentration of the normal substrate by competing with it for the enzymes.

Using amino acid analogues, Thompson reported that analogues of glycine (amino methane sulfonic acid), valine (α -amino isobutane sulfonic acid), phenylalanine (α -amino phenylmethane sulfonic acid), and methionine (methoxinine) suppressed the replication of vaccinia virus in Maitland-type chick embryo tissue cultures (4). In his conclusion, Thompson stated that, although these compounds may be toxic for tissues, it is possible that the inhibition of virus growth is due to an interference with the utilization of amino acids by the infected cells. The specificity of amino acid analogues was later shown by Thompson and Wilkin, who reversed the inhibitory effect of β -2-thienylalanine on vaccinia virus replication by the addition of an excess of phenylalanine (5). Furusawa et al. also demonstrated the requirement of tryptophan for vaccinia virus in HeLa cells by using 5-methyltryptophan (6,7). The requirement of methionine for poliovirus replication has been shown with its analogue, ethionine, in monkey testes cultures (8) and in HeLa and monkey kidney cells (9); and for phenylalanine with β -2-thienylalanine in monkey testes cultures (8).

It is evident from the reports listed and from other published studies that an essential amino acid, such as methionine, is commonly required for the replication of many viruses, such as vaccinia, polio, influenza (10) and psittacosis (11).

The importance of assessing the general cytotoxicity of analogues has been stressed by Morgan, who stated that any interference with normal cellular proliferation in a tissue culture will reduce viral growth by reducing the population of susceptible cells (11). For example, methionine is an essential amino acid for continuous growth of cell cultures, and its omission from tissue culture media results in growth inhibition. Accordingly, it becomes difficult to ascribe any degree of specificity to the action of an antimetabolite on viral biosynthesis.

In view of this consideration, the present study was aimed at evaluating the cytotoxicity of methionine and glycine analogues by measuring the cell growth as well as the replication of vaccinia virus in terms of virus yield per cell in nutritionally deficient cultures.

The effect of the methionine analogue, l-ethionine, and the glycine analogue, glycine methylester, on the growth of human Chang liver cells and on vaccinia virus replication was investigated in two parallel sets of tube cultures, as has been described previously (9). The cytotoxicity of the compounds was measured by changes in cell morphology and by growth inhibition. The growth was expressed as increases of cell protein per culture during specified periods of incubation. The amount of cell protein was determined with Folin-Ciocalteu reagent, according to the method of Oyama and Eagle (12).

The analogue-treated cultures were infected with vaccinia virus (strain 971 H) six hours after the addition of the analogue. Virus-infected cultures were examined microscopically, and were scored according to the progressive cytopathogenic changes. The magnitude of virus replication was determined by harvesting the virus-infected cultures 24, 48 and 72 hours after infection. In order to titrate the viral progeny, virus was liberated from the

cells by repeated freezing and thawing, and its infectivity in human Chang liver cells was measured by the tube titration method (13). The highest dilution of virus inducing infection in 50% of the cultures was expressed as the TCID₅₀ (tissue culture infective dose 50%), and the value was calculated from the cytopathogenicity results obtained from 4 to 5 cultures at each virus dilution, according to the method of Reed and Muench (14).

When l-ethionine was incorporated in Eagle's medium (15) containing methionine and 10% of serum at 2 mM and 4 mM levels, there was no detectable morphological cytotoxicity for Chang liver cells, but their growth was inhibited, as shown in Figure 1. The growth inhibition was progressive and proportional to the concentrations of ethionine, as well as to the length of incubation. In the presence of 2 mM of l-ethionine, the total cell protein per culture after incubation for 48 hours was 82% of the control value, and with 4 mM of ethionine, it was 65%. The results are illustrated on the left side of Figure 1.

The magnitude of virus replication is indicated in Figure 1. The infectivity titer of progeny (TCID₅₀) was reduced more than 1.0 logarithm in both ethionine-treated cultures. When the virus yield is expressed as percentages of the control, the virus yields in the ethionine-treated cultures were less than 10% of the control yields.

The reduction in the amount of virus per culture may be dependent on two factors: the number of cells present in the ethionine-treated cultures, and the reduced capacity of the cells for viral biosynthesis. Since the ethionine-treated cultures contained fewer cells, as indicated by the reduced amount of cell protein present in those cultures, the magnitude of viral biosynthesis per individual cell may be partly estimated by expressing the virus yield per microgram of cell protein in the corresponding cultures, as it has been shown that the amount of cell protein is proportional to the number of cells (12). These ratios are indicated on the right side of Figure 1, and show that the ethionine-treated cells yielded less than 15% of the controls. Therefore, the reduction in virus yield is primarily an effect of l-ethionine on the mechanism of viral biosynthesis, and is not due to the slightly reduced number of cells present in the ethionine-treated cultures.

The specific interference of ethionine with the utilization of methionine is indicated in Figure 2. The inhibitory effect of l-ethionine on the growth of human Chang liver cells was partly reversed by the addition of 6 mM of l-methionine.

The partial reversal of the inhibitory effect of l-ethionine on the replication of vaccinia virus by the addition of an excess of l-methionine is indicated in Figure 3. The results are expressed as percentages of the control values. The virus yield with 2 mM of ethionine was 2% of the control values, but in combination with 6 mM of methionine, it was 18% of the control. Under the same conditions, methionine alone, at 6 mM level, had a marked inhibitory effect on the virus replication, although it did not reduce the growth of cells significantly. The virus yield per microgram of protein was only 17% of the control.

Applying the same testing procedures, we also investigated the effects of a non-essential amino acid, glycine, and its analogue, glycine methylester. The results are summarized in Figure 4. In the presence of 1 mM of glycine, the growth of Chang liver cells was slightly inhibited without any evidence of morphological cytopathogenicity. The cell protein per culture was 84% of the control. At the same concentration, glycine inhibited vaccinia virus replication markedly, and the yield was only 12% of the control yield. The analogue, glycine methylester, at 0.5 mM per culture, was highly inhibitory for the cell growth and for virus replication. As the inhibitory effect of glycine methylester on cell growth and virus replication was not reversed by the addition of glycine, the results suggest that glycine methylester inhibits cell growth and virus replication by mechanisms other than a simple competition with the utilization of glycine.

From this data it is evident that either an excess of methionine, one of the essential amino acids, or an excess of glycine, one of the non-essential amino acids, is highly inhibitory for vaccinia virus biosynthesis. Our preliminary studies with increased amounts of leucine indicate the same response. These findings suggest the possibility that the balance of amino acids in the intracellular amino acid pool may be important for proper virus replication, as it is for the maintenance and growth of higher organisms. This possibility seems to agree with Hill's conclusion that certain nutrients, in amounts greater than those required by healthy animals, may in some instances increase resistance to infection, although an indiscriminate increase in nutrients may not only have no effect, but may actually decrease resistance to infections (16).

The second part of our study correlates these results obtained with ethionine in vaccinia virus infections in cell cultures with those obtained with ethionine in albino rabbits maintained on Purina rabbit chow diet.

To induce a methionine deficiency, rabbits were daily injected intraperitoneally with 2.7 to 2.8 mg of ethionine per kg of body weight for the first 5 days of this study. On the 6th day, the concentration of ethionine was doubled and ranged from 5.5 to 5.7 mg per kg. This dosage was continued until the 14th day of the experiment. On the 8th day after the first treatment, the animals were injected intradermally with 0.1 ml of three different vaccinia virus dilutions, which contained 5, 50 and 500 rabbit ID₅₀'s per 0.1 ml, respectively. Each dilution was injected into two separate areas on the back of each rabbit.

The effects of ethionine treatment on the vaccinia virus infection are summarized in Figure 5. On the second day after infection, the vaccinia virus-induced skin lesions appeared as inflamed eruptions. The lesions were classified as 1+ to 6+ exanthemas according to the size and intensity of the reactions, and the magnitude is illustrated in the upper part of Figure 5. The scores of vaccinia skin lesions show that the onset was delayed for one day in the ethionine-treated rabbits, and the exanthemas were generally less pronounced than in the control animals.

The rectal temperatures of vaccinia-infected rabbits are indicated in the middle section of Figure 5. All infected rabbits showed an increased body temperature during the infection period. In the ethionine-treated rabbits, the body temperature rose by $1.1F^{\circ}$, returning to the normal level on the 3rd day after infection, whereas in the control rabbits, it was increased by $2.7F^{\circ}$ and was still $2.1F^{\circ}$ above the normal level on the third day. The results of skin lesions and body temperature indicate that the vaccinia infection in the ethionine-treated rabbits was less severe than in the control animals.

Changes in the body weight of rabbits during the experiments are indicated in the lower portion of Figure 5. All rabbits lost some weight during the acute stage of vaccinia virus infection.

In order to assess the immune responses of rabbits to vaccinia infection, a virus neutralization test was performed, using the serum harvested 14 days after infection. The titer of neutralizing antibodies was determined by mixing 1 ml of each serum dilution in Eagle's medium with 0.1 ml of a virus dilution containing 50 TCID₅₀'s. The antiserum-virus mixtures were incubated at 37° for 45 minutes, and then 1 ml was added to Chang lever tube cultures. The serum neutralization titer of the control group was dilution 1/128, and of the ethionine-treated group, the titer was dilution 1/32; accordingly, a four-fold titer difference.

The protective action of antibody in vivo against reinfection with vaccinia virus was estimated by infecting the previously infected rabbits and some non-infected rabbits with a virus dilution which contained 10 times more virus than was used during the first infection. The previously infected control rabbits were completely protected against reinfection with vaccinia virus, whereas the ethionine-treated previously infected rabbits showed only a partial protection. Accordingly, the results of the protection test correlated well with the titer of neutralizing antibody determined in vitro. The reduced titer of antibody in the ethionine-treated rabbits may be explained by an inhibitory effect of ethionine on the antibody-producing system.

In summary, both analogues of methionine and glycine suppressed vaccinia virus replication in human liver cells significantly more than they inhibited the growth of cells. The fact that an excess as well as a deficiency of both methionine and glycine inhibited virus replication more than cell growth indicated that the balance of the intracellular amino acid pool may affect the magnitude of viral biosynthesis.

The inhibitory effect of l-ethionine on vaccinia virus replication in cell cultures correlated well with the results obtained in the rabbits. The cell culture testing system, therefore, proved a useful tool for comparing the effects of single nutrient deficiencies or excesses on the growth of cells and on virus replication.

SUMMARY

Studies on the interaction of nutrition and infections have shown that some viral infections are less severe with certain types of nutritional deficiencies. The methods of tissue and cell culture offer some quantitative advantages for evaluating the effects of single nutrients upon cell growth and viral replication. The effects of methionine and glycine deficiencies were determined by using their analogues.

L-ethionine and glycine methylester suppressed the replication of vaccinia virus in human Chang liver cells significantly more than they inhibited the growth of cells. The inhibitory effect of ethionine was partly reversed by the addition of an excess of methionine, but the effect of glycine methylester was not reversible by an excess of glycine. Since an excess as well as a deficiency of both methionine and glycine inhibited vaccinia virus replication more than cell growth, it appears that the balance of the intracellular amino acid pool may affect the magnitude of viral biosynthesis.

As ethionine also reduced vaccinia virus infection and antibody level in the rabbit, the cell culture testing system proved a useful tool for comparing the effects of single nutrient deficiencies on cell growth and virus replication.

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FIGURE 1

EFFECT OF β -ETHIONINE ON GROWTH OF HUMAN CHANG LIVER CELLS AND ON VACCINIA VIRUS REPLICATION

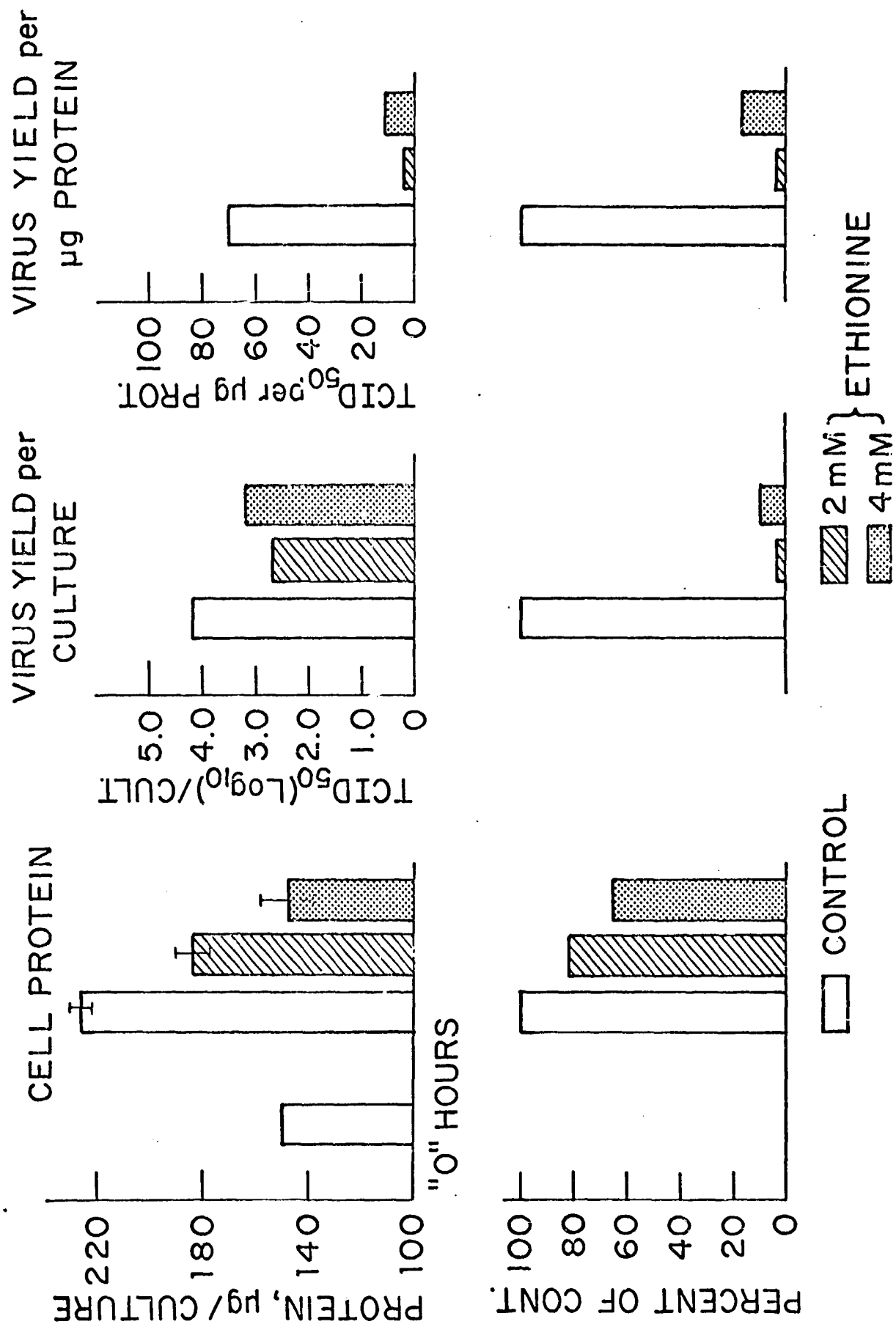


FIGURE 2

REVERSAL OF INHIBITORY EFFECT OF β -ETHIONINE ON GROWTH OF LIVER CELLS BY β -METHIONINE

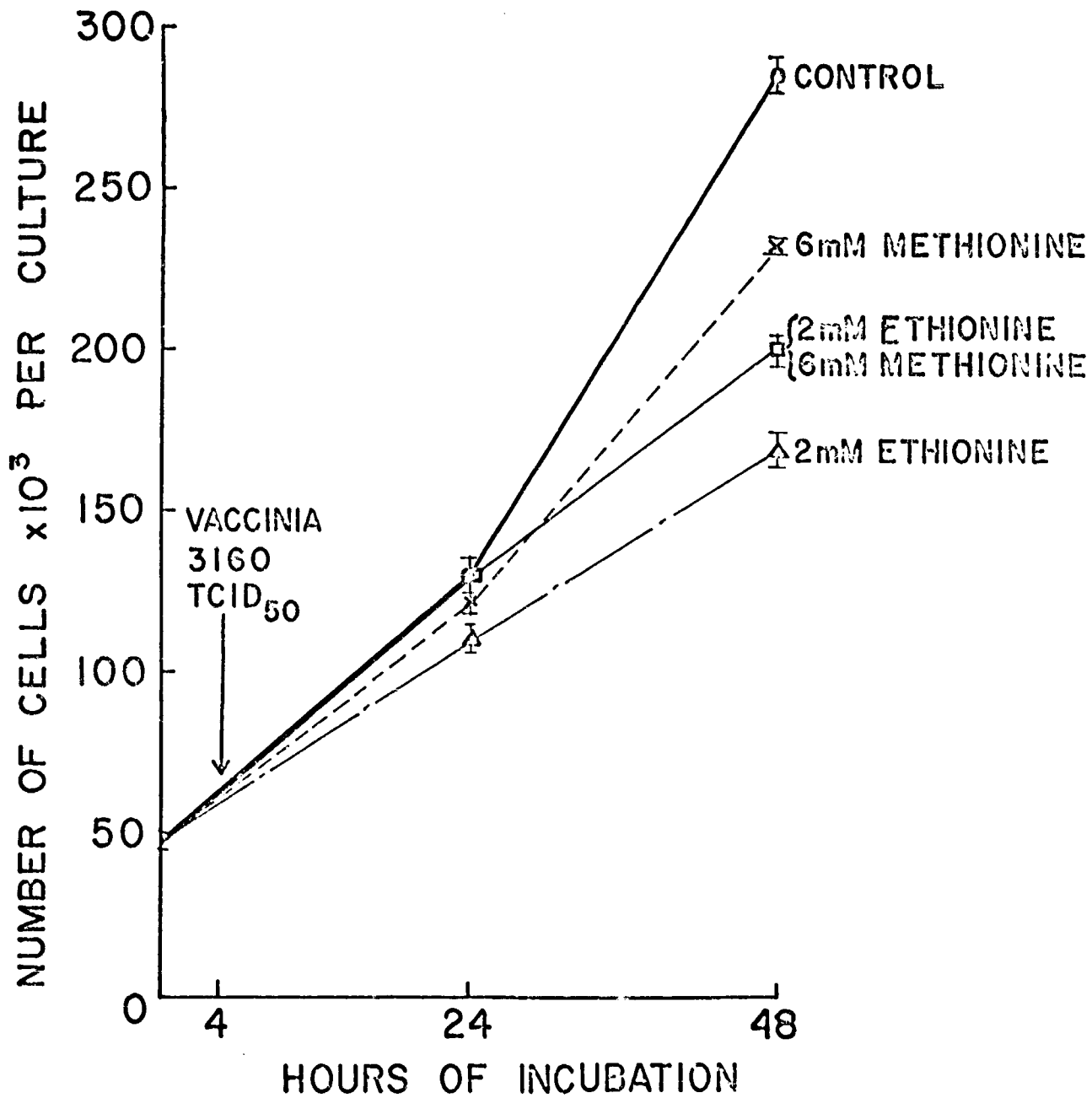


FIGURE 3
 REVERSAL OF INHIBITORY EFFECT OF β -ETHIONINE
 ON REPLICATION OF VACCINIA VIRUS

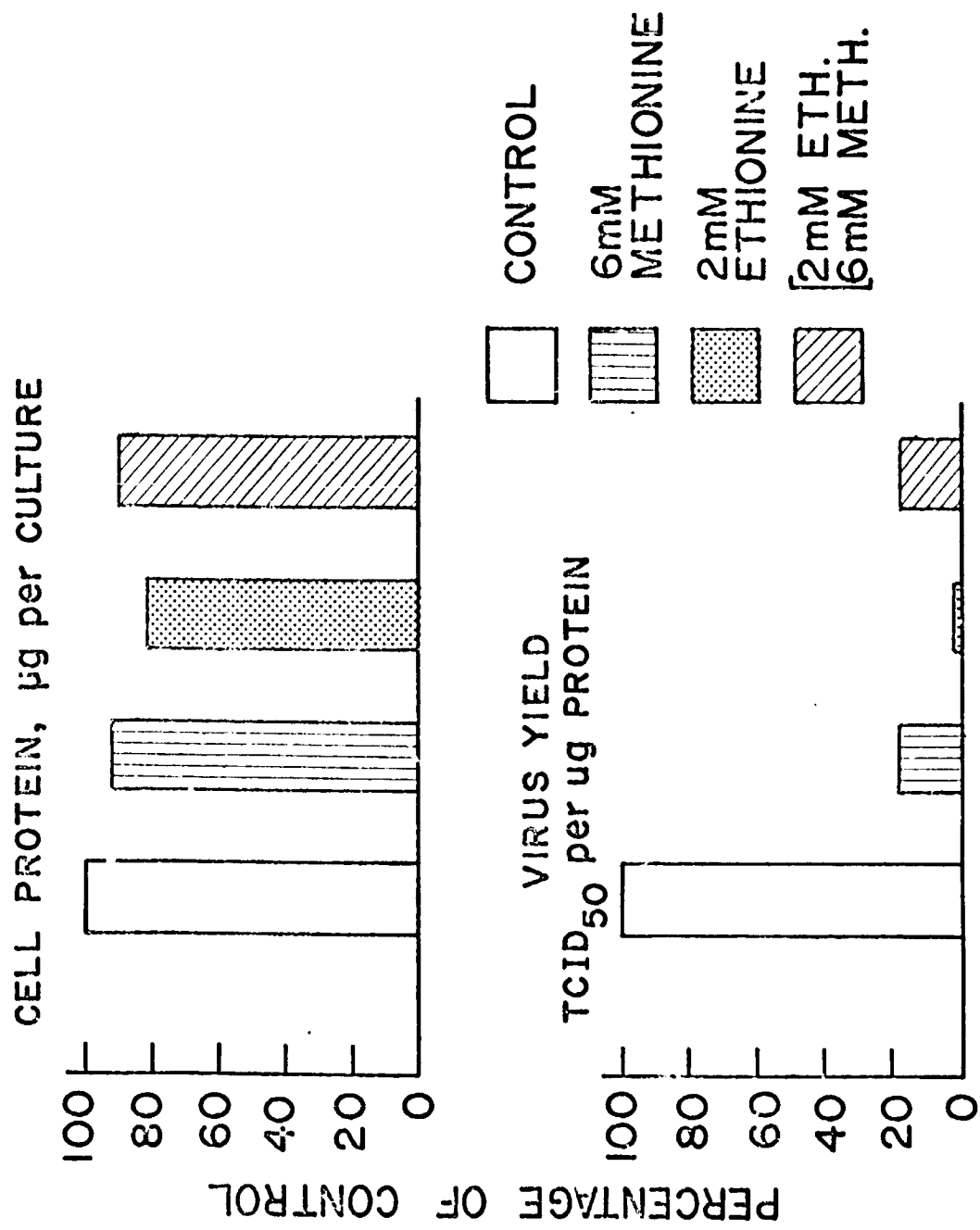


FIGURE 5

VACCINIA VIRUS INFECTION IN ETHIONINE
TREATED RABBITS

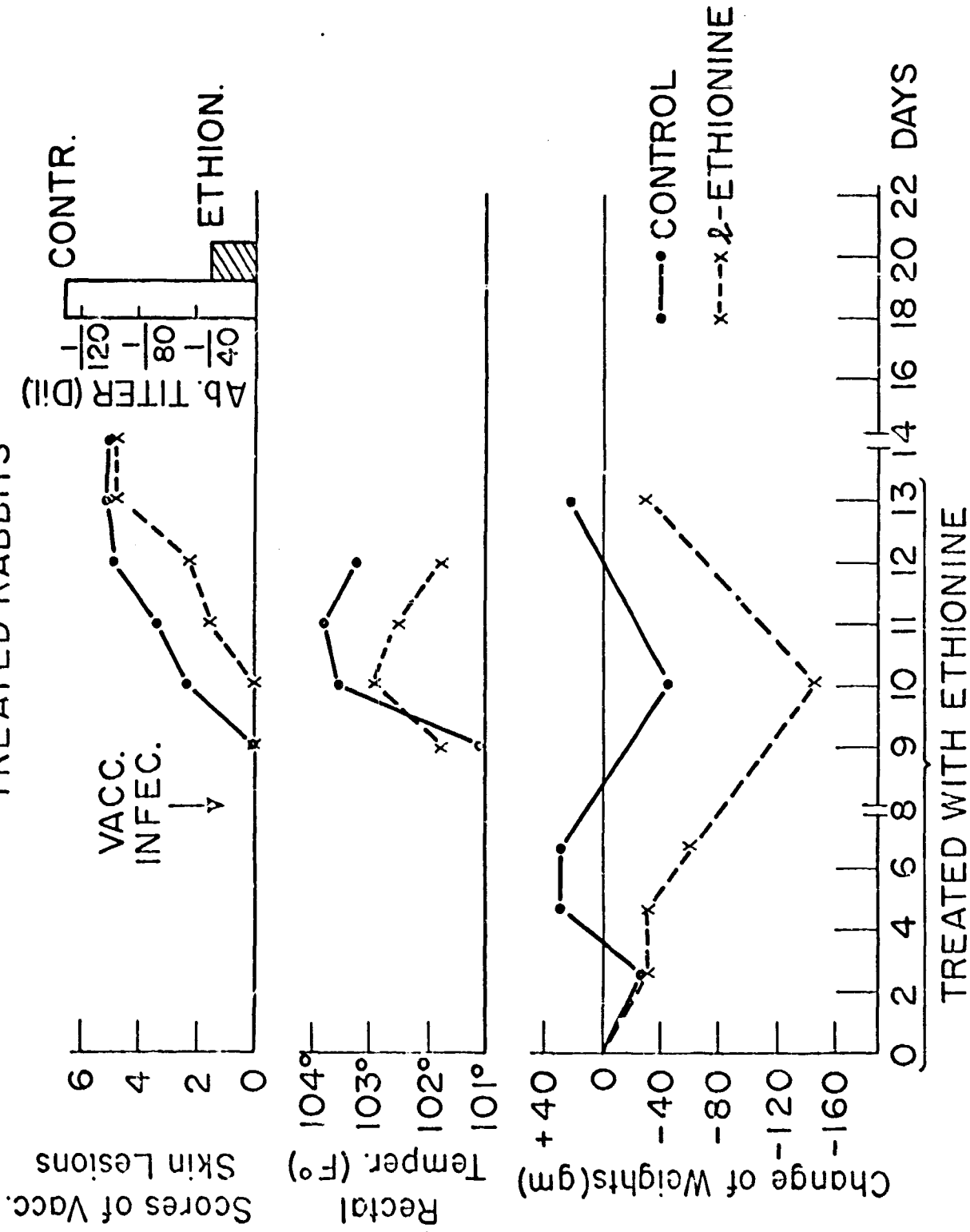
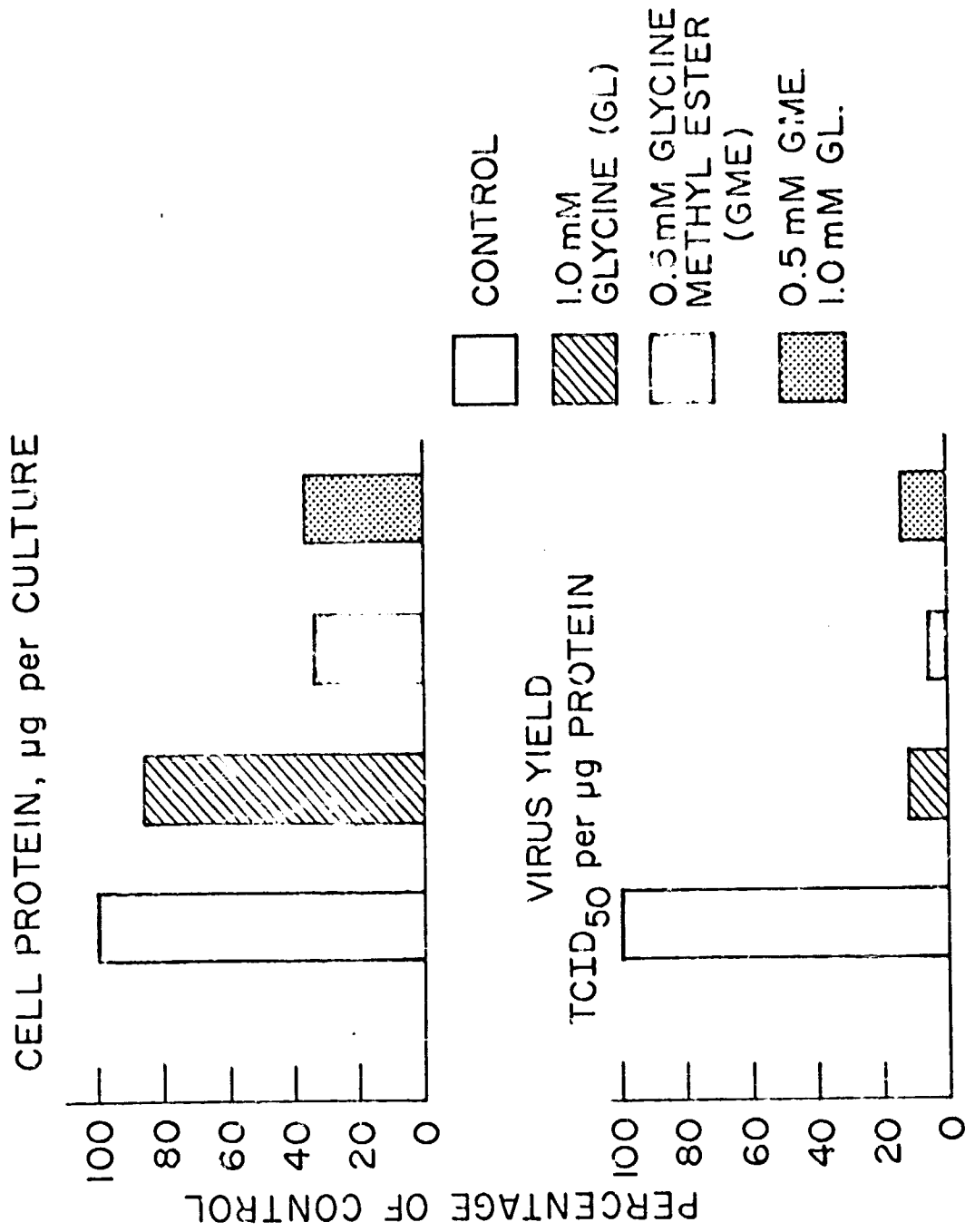


FIGURE 4
 EFFECT OF GLYCINE AND GLYCINE METHYL ESTER
 ON GROWTH OF CELLS AND VACCINIA VIRUS



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13 ABSTRACT I. <u>Toxins Produced by Microorganisms</u> - The cytotoxicity of <i>Staphylococcal enterotoxin B</i> to cells of human embryonic intestine is markedly reduced by trypsin. The temporary resistance increases proportionally with increased time of exposure to trypsin and lasts for 48 hours. It appears that trypsin inactivates specific cell receptors which may be essential for interaction with enterotoxin. II. <u>Selected Toxic Substances</u> - Organochlorine and organophosphorus compounds (inhibitors of acetylcholinesterases) DDT, chlordane, Kelthane ^R , Dipterex ^R , malathion, and Karathane ^R at subtoxic concentrations inhibit vaccinia virus replication in human Chang liver cells. The replication of poliovirus is inhibited only by chlordane and malathion, whereas Kelthane and Karathane increase the poliovirus yields. III. <u>Nutritional Factors in Viral Infections</u> - A deficiency of methionine and glycine induced by their analogues, and a deficiency of leucine in the medium suppress vaccinia virus replication significantly more than they inhibit the growth of cells. The replication of virus is also inhibited when the medium contains an excess of either methionine, glycine, leucine, isoleucine, or valine. The inhibitory effect of methionine on virus replication in cell cultures correlates well with the results obtained with vaccinia virus infection in the rabbit.		

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KEY WORDS	LINK A		LINK B		LINK C	
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