THE EFFECT OF DIPHTHERIA TOXIN ON THE METABOLISM OF HeLa CELLS*

By NORMAN STRAUSS, Ph.D.,‡, § and EDELMIRA D. HENDEE

(From the Department of Microbiology, New York University-Bellevue Medical Center, New York)

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From the time of Rous and Yersin, who first reported the toxicity of sterile filtrates from cultures of the diphtheria bacillus some 70 years ago, the means by which diphtheria toxin exerts its lethal effect has not been discovered. Following the injection of large doses of diphtheria toxin into susceptible animals there is a prolonged latent period of many hours during which no ill effect of any kind, either morphological or biochemical, can be detected. It is now generally agreed (1, 2) that all of the familiar signs of diphtheria intoxication including gross and microscopic morphological damage to tissues, increased resistance of the animal to insulin (Corkill (3)), reduced capacity to synthesize carbohydrates (Cross and Holmes (4)), or to metabolize lactic acid (Dawson and Holmes (5)) and decreased stores of muscle phosphocreatine (Pinchot and Bloom, (6)) are all secondary effects which follow by many hours a primary injury the nature of which is unknown. Peters and Cunningham (7) examined various oxidative enzyme systems which had been treated with toxin *in vitro*, but could find no untoward effects. Pappenheimer and Williams (8), using the metamorphosing silkworm *Platysamia cecropia* as the susceptible animal, found that only those tissues characterized by an active succinoxidase system, or undergoing rapid growth and development dependent on the succinoxidase system, were sensitive to the action of toxin; whereas tissues which were deficient in the succinoxidase system were insensitive.

With the advent of improved tissue culture methods and consequent stabilization of certain mammalian cell lines *in vitro*, more homogeneous cell populations became available for use in studying the effects of toxin. Lennox and Kaplan (9) and Placido Sousa and Evans (10) found various cell lines to be susceptible to diphtheria toxin.

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‡ Present address: Department of Microbiology, Yale University School of Medicine, New Haven.

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In the work to be presented, we have used a stabilized line of human epithelial carcinoma cells (HeLa) for studying the kinetics of intoxication, and the effects of diphtheria toxin on certain metabolic systems, in particular its effect on protein synthesis.

Materials and Methods

Cells.—A human epithelial carcinoma, HeLa, strain S-3 (11, 12) was grown on a glass surface at 37°C. in a partially defined medium. For metabolic experiments, culture bottles were heavily seeded with HeLa cells and harvested 48 hours later by subjecting them to the action of a 0.05 per cent trypsin solution (Nutrition Biochemicals Corp., Cleveland, 1–300). The cells were then washed by centrifugation several times with either a modified Hanks' solution (saline A) (13) or complete medium containing 2 per cent normal horse serum, and resuspended in appropriate solutions.

Media.—Experiments on respiration were carried out using Puck's medium (13) containing 20 per cent normal horse serum. Later work, involving radioisotopes was carried out using Eagle's medium (14) containing 2 per cent normal horse serum. The serum used for all experiments was taken from a single horse and was found to contain 0.01 to 0.10 units of antitoxin per ml. This serum was dialyzed in the cold for 4 hours, with stirring, against two changes of saline, in order to remove any free methionine that might be present.

Determination of Cell Densities.—Cell counts were carried out according to the method of Sanford and associates (15) as modified by Oyama and Eagle (16). Equal volumes of cell suspension and stain were mixed and allowed to stand at room temperature for at least 15 minutes. The stained nuclei were then enumerated in a Spencer hemocytometer. In each case, eight separate preparations were made, counted, and their average taken.

Protein was determined by the method of Lowry and associates (17) as modified by Oyama and Eagle (16). The standard used was a solution of Armour fraction V bovine serum albumen for which the nitrogen content had been determined by micro-Kjeldahl analysis.

Lactic Acid.—Lactic acid was determined by the method of Barker and Summerson (18).

Aerobic Respiration.—Oxygen consumption was followed by conventional Warburg techniques. Carbon dioxide partial pressure was maintained by the use of a "CO_2 buffer" (19, 20) which was composed of a solution of diethanolamine, thiourea, and potassium bicarbonate which had been equilibrated with a 2 per cent CO_2 -98 per cent N_2 gas mixture. Cells were suspended to a density of about 10^6 per ml. in complete medium. To one-half of this suspension was added diphtheria toxin to a level of 3 Lf per ml. To the other half was added as a control, the same amount of toxin in combination with excess antitoxin. Each Warburg vessel received 0.3 ml. of "CO_2 buffer" in the side arm and center well respectively, 1.3 ml. of cell suspension in complete medium (which contained toxin or toxin-antitoxin mixtures) in the main compartment, and the volume was adjusted to 2.0 ml. with complete medium. The flasks were then placed in the water bath at 37°C. and flushed for 25 minutes with a 2 per cent CO_2-98 per cent air mixture. At zero time the stop-cocks were closed. Toxin was always added to the cell suspensions 40 minutes prior to zero time. Duplicate flasks were removed at intervals for protein determinations.

S^{35}-Methionine Incorporation.—All suspensions were prepared in Eagle's medium containing 2 per cent horse serum, and lacking methionine. Cell densities were adjusted to 1 to 2×10^5 per ml. The suspended cells were dispensed in 15 ml. portions to 50 ml. flasks and equilibrated at 37°C. in a rotary water bath at 130 strokes per minute for 20 minutes. At zero time S^{35}-L-methionine was added to give a final concentration of 9 micrograms per ml. (6×10^{-5} M) and about 6×10^4 counts per minute (C.P.M.) per ml. as measured on a thin window

gas flow counter. Aliquots of suspension were removed, diluted in saline A, retained on Millipore filters, and washed first with saline and then with 5 per cent trichloroacetic acid (TCA) containing 150 micrograms of non-radioactive methionine per ml. The filter pads were mounted on copper discs, dried, and counted. The counting rate was calculated from the time necessary for the sample to undergo at least 1600 disintegrations. The omission of the TCA wash resulted in a lowered counting rate of samples which contained more than 10^5 cells. This was found to be due to an increased self-absorption of radiation. All determinations were done in duplicate The average deviation of the determinations was ± 4 per cent.

Diphtheria Toxin.—Toxin was prepared and purified by the method of Yoneda (21). The toxin contained 6300 Lf units per ml., and 50 guinea pig M.L.D. per Lf. It was diluted in complete medium to a concentration of 63 Lf per ml. It is to be emphasized at this point that the immunological unit is used here as a measure of toxicity only for convenience.

Diphtheria Antitoxin.—Concentrated horse antitoxic globulin (Massachusetts Department of Health) was dialyzed exhaustively in the cold to remove preservative. It was then diluted so as to contain 500 units per ml. In concentrations as high as 75 units per ml. it was found to have no toxic effect on HeLa cells.

RESULTS

Cytopathogenic Effects of Toxin.—While no detailed studies were made, it was observed that after several hours, intoxicated[1] cells became somewhat rounded and granular, and then assumed a raspberry contour due to the appearance of numerous swellings on the cell surface. Eventually the cell outlines disappeared, leaving an apparently intact nucleus surrounded by shreds of cytoplasm. In the presence of toxin concentrations of 0.3 Lf per ml. and higher, cytopathogenic effects first became visible in 4 hours in a few cells. The numbers of visibly damaged cells increased rapidly and by the 7th hour after the administration of toxin large numbers of cells were destroyed.

Glycolysis.—The glycolytic process was followed by determining the lactic acid formed per unit time.

Cells were suspended to a density of about 10^6 per ml. in Puck's medium containing 20 per cent horse serum. The suspension was divided into two parts, toxin added to one part to give a final concentration of 3 Lf per ml., and toxin added in the same amount to the other flask in combination with excess antitoxin.[2] Samples were removed at 1 hour intervals for lactic acid determinations.

It can be seen from Fig. 1 that toxin causes no appreciable inhibition of lactic acid formation prior to 5 hours, at which time deterioration of the intoxicated cells begins. The fact that glycolysis is unaffected prior to this time would appear to eliminate this process as the primary target of toxin action.

[1] An intoxicated cell is here considered to be one which cannot be saved by the use of antitoxin.

[2] In all the experiments to be described, including those on aerobic respiration and radioisotope incorporation, it was found that control suspensions to which toxin was added in combination with excess antitoxin behaved in an identical manner, in all cases, as did untreated cell suspensions.

In this connection, it is of interest that Levy and Baron (22) have recently reported an increase in the rate of lactic acid formation by intoxicated monkey kidney cells. No data were presented however.

Effect of Toxin on Oxygen Consumption and Growth.—A comparison of normal and intoxicated cells with respect to oxygen uptake and concomitant protein synthesis is shown in Fig. 2.

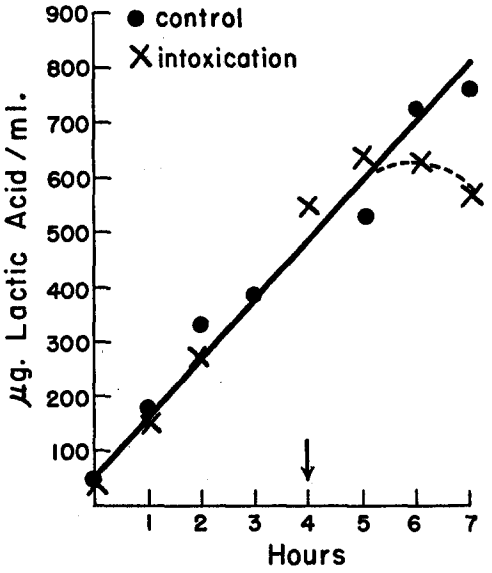

FIG. 1. The formation of lactic acid by normal and intoxicated HeLa cells in complete growth medium. Cultures were trypsinized, washed and suspended in complete medium as singly dispersed cells to a level of 6.4×10^5 per ml. At zero time minus 0.67 hours, toxin was added to one flask in a final concentration of 3.0 Lf/ml. Control flasks received the same amount of toxin which had been preincubated with excess antitoxin. The arrow indicates the beginning of visible cell damage.

Cell suspensions in complete growth medium were distributed into each of eight flasks. Four of the flasks received toxin, and four received toxin neutralized with excess antitoxin. After suitable equilibration, the stop-cocks were closed, and one flask of each series removed for protein determination. Readings of oxygen consumption were made from the remaining flasks and the average oxygen uptake for each series of flasks calculated at intervals. At 4, 8, and 12 hours one flask of each series was removed for protein determination. Readings of oxygen consumption were made from the remaining flasks until the 12th hour.

It is clear that within the period of observation small but significant differences, as judged by repeated trial, are evident in oxygen uptake and protein synthesis. The theoretical curve for oxygen uptake by a growing culture with a generation time of 22 hours was calculated using the equation:

$$\text{Total oxygen consumed} = K[e^{kt} - 1]$$

in which K is a constant which includes P_0, initial total cell protein; k, the growth constant, and a proportionality constant relating O_2 consumption and total cell protein. The theoretical curve for protein synthesis by an exponentially growing cell population was calculated using the equation:

$$P = P_0 e^{kt}$$

in which P is the amount of protein at time t, and P_0 is the initial protein. It can be seen that the experimental and theoretical curves are in good agreement.

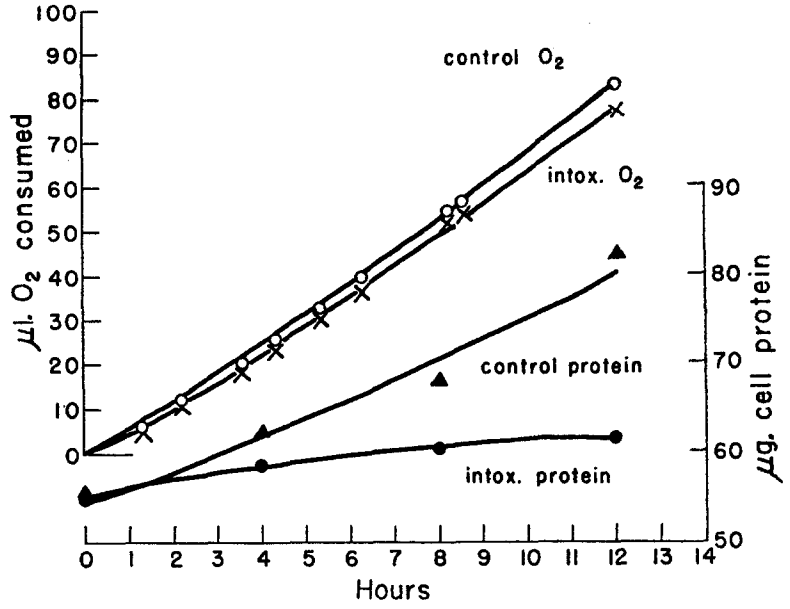

FIG. 2. Respiration and protein synthesis of normal and intoxicated cells in complete growth medium. Toxin concentration was 3.4 Lf/ml. The cell concentration was adjusted to 5×10^5/ml. The points represent experimental data. The theoretical consumption of O_2 and yields of protein, expected of *the controls*, are shown by solid lines (see text).

The results obtained by these relatively insensitive methods suggested an early inhibition of protein synthesis, and for this reason a study of intoxication using tracers was indicated.

Incorporation of S^{35}-Methionine into Protein.—

Cells growing at 37°C. were permitted to incorporate S^{35}-methionine for 6 hours. At the end of this time, the cells were removed from the glass surface with versene (0.005 M) rather than trypsin. The cells were suspended in a known volume of saline A. Aliquots of this suspension were filtered and the radioactivity determined as described. Aliquots were also taken for protein determinations. The remaining suspension was then subjected to fractionation by the method of Schneider (23) as modified by Siekevitz (24): the cells were extracted

TABLE I

Comparison of the S^{35}-Methionine Content of Filtered and Fractionated Cells

Treatment of cells	Counts per minute per ml.	Micrograms protein per ml.	Specfic Activity (C.P.M./μg. protein)
Filtered*	3030 ± 200‡	(195)§	—
Non-fractionated‖	2940 ± 48	195 ± 4.3	15.1
Fractionated¶	2530 ± 80	168 ± 2.9	15.1

* A 1.0 ml. aliquot of cell suspension was diluted in 20 ml. of saline, retained on a Millipore filter, washed once with saline and once with 5 per cent trichloroacetic acid containing 150 micrograms of cold methionine per ml. The radioactivity was determined as described in the text.

‡ Mean deviation fron the mean.

§ Assumed from the value obtained for non-fractionated cells.

‖ A 1.0 ml. aliquot of the same suspension was centrifuged, washed several times, and the cells dissolved in a known volume of 0.1 N NAOH. Aliquots were taken for protein and radioactivity determinations.

¶ Fractionated by the method of Schneider as modified by Seikevitz (23, 24). The hot TCA-insoluble fraction was dissolved in a known volume of 0.1 N NAOH. Aliquots were taken for protein and radioactivity determinations.

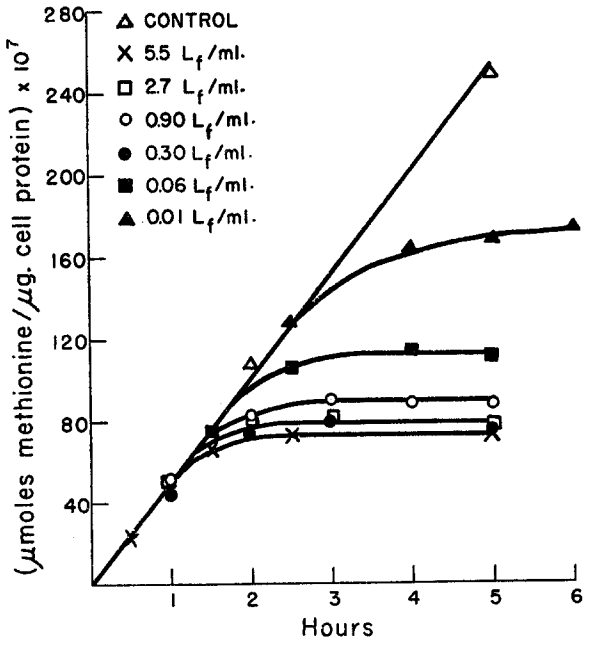

FIG. 3. The effect of toxin concentration on the incorporation of methionine into protein. Conditions described in text.

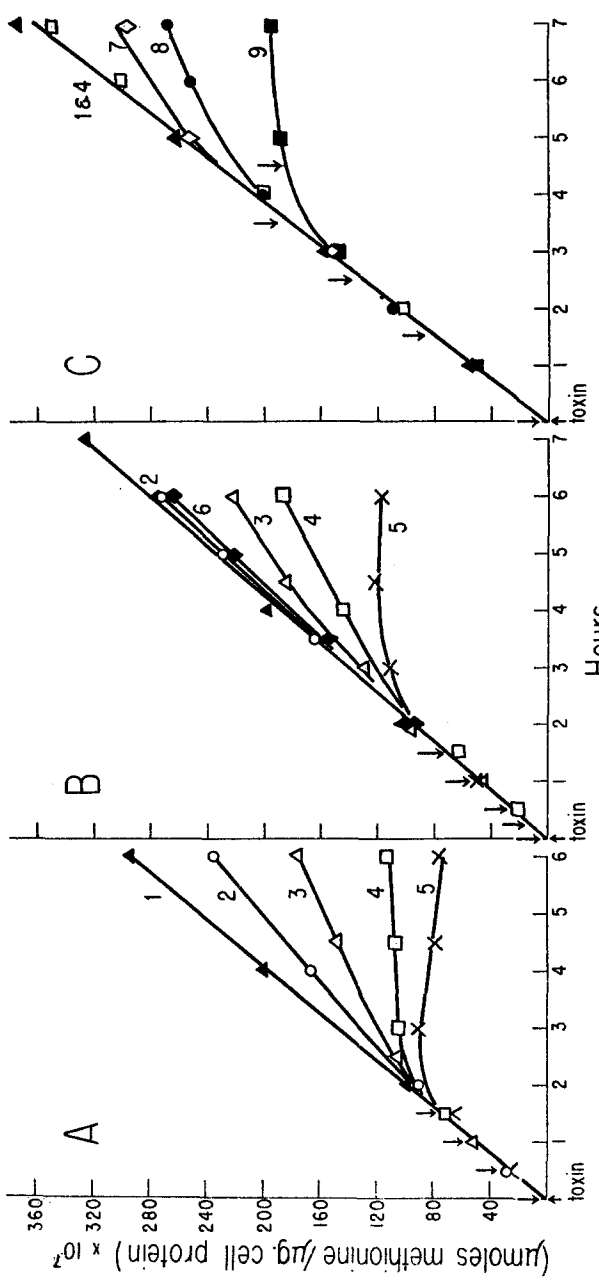

FIG. 4. The effect of addition of antitoxin to HeLa cells during the "lag."
A. 0.32 Lf per ml.
B. 0.06 Lf per ml.
C. 0.01 Lf per ml.
Toxin was added at zero time. Arrows indicate time of addition of antitoxin (1) control; (2) Antitoxin (AT) at 0.5 hour; (3) AT at 1.0 hour; (4) AT at 1.5 hours; (5) no AT; (6) AT at 0.25 hour; (7) AT at 2.5 hours; (8) AT at 3.5 hours; (9) AT at 4.5 hours.

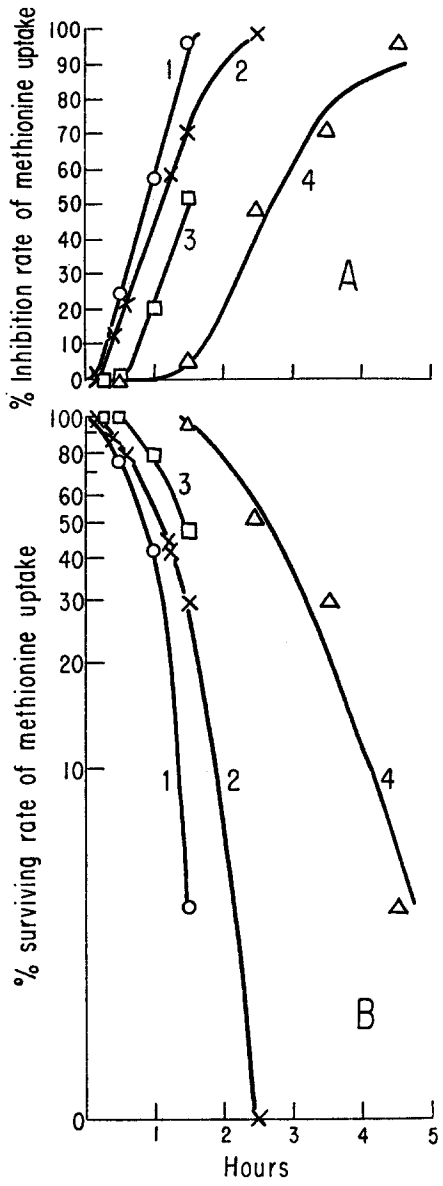

FIG. 5.

A. The relation between per cent inhibition of rate of methionine uptake and time of exposure to different toxin concentrations.

B. The relation between surviving rate of methionine uptake and time of exposure to different toxin concentrations.

(1) 0.3 Lf per ml.; (2) 0.1 Lf per ml.; (3) 0.06 Lf per ml.; (4) 0.01 Lf per ml.

with cold 5 per cent TCA containing non-radioactive methionine, with hot 5 per cent TCA, and then with organic solvents. The residue was dissolved in a known volume of 0.1 N NaOH, and aliquots taken for radioactivity and protein determinations. The specific activities of the non-fractionated and fractionated cells were calculated and compared.

Justification for the assumption that virtually all methionine incorporated into growing cells is in the form of protein is seen in Table I. As can be seen, no change was evident in the specific radioactivity of the protein following fractionation.

The Effect of Toxin on S^{35}-Methionine Incorporation.—The incorporation of S^{35}-methionine into normal cells under the conditions prevailing can be considered to be linear during the time of observation (6 to 7 hours). In Fig. 3 are shown the effects of various concentrations of diphtheria toxin on the uptake of S^{35}-methionine. The inhibition of incorporation of this amino acid by toxin is striking and abruptly follows a period of normal uptake. Below 0.3 Lf per ml. the length of time which precedes this sudden break in incorporation is inversely related to the toxin concentration. The fact that this seemingly normal period attains a minimum value of 1 hour in the presence of levels of toxin greater than 0.3 Lf per ml. indicates the involvement of a system of limited capacity; *i.e.*, a system which can be saturated. Despite the fact that protein synthesis is arrested completely in 2 hours in the presence of saturating concentrations of toxin, the cells appear normal until the 4th hour. At this time the first signs of morphological damage become evident in a few cells. By the 7th hour many of the cells are completely disrupted.[3] When toxin concentrations lower than 0.3 Lf per ml. are used, the time of appearance of inhibition of methionine uptake is delayed, but the inhibition still reaches 100 per cent with toxin concentrations as low as 0.01 Lf per ml. Lower concentrations were not used because of the presence in the horse serum of a small amount of antitoxin.

Kinetics of Intoxication.—In order to examine the kinetics of intoxication prior to the appearance of inhibition of S^{35}-methionine uptake, antitoxin in tenfold excess was added to cell suspensions at various times after the addition of different concentrations of toxin. The results, shown in Figs. 4 A, to 4 C, clearly indicate that antitoxin can arrest the action of the toxin if added early enough. Thus the intoxication step can be isolated from the reversible step(s) which precede it. The data comprising Fig. 4 show that the period required for toxin uptake is constant at a given toxin concentration regardless of the extent of intoxication of the population. A plot of the per cent inhibition of the rate of methionine uptake against time of exposure to toxin (*i.e.* before addition of antitoxin) is shown in Fig. 5 A. Such S-shaped curves are characteristic of

[3] This results in the loss of labelled protein during the process of filtration, and accounts for the occasionally observed slight decline in S^{35}-methionine content of cells which have ceased to incorporate methionine.

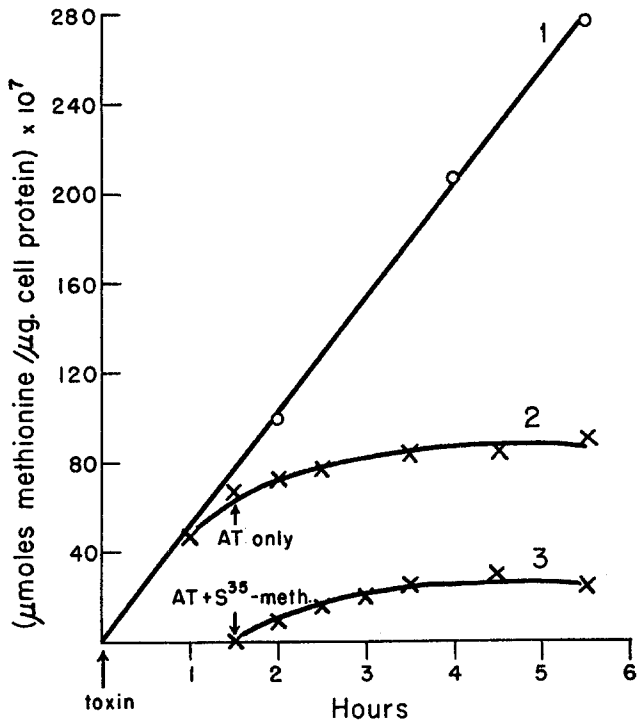

FIG. 6. Effect of addition of S^{35}-methionine to a fully intoxicated cell suspension.
(1) Non-intoxicated control.
(2) S^{35}-methionine and toxin (final concentration 0.3 Lf per ml.) added at zero time. Antitoxin added at 1.5 hours.
(3) Toxin added at zero time. S^{35}-methionine and antitoxin added at 1.5 hours.

several distinctly different types of events (*e.g.* autocatalysis, probability, or even first order killing preceded by a lag). A replot of the rate of methionine incorporation (expressed as per cent of normal) as a function of time of exposure to toxin serves to characterize the kinetics somewhat further (Fig. 5 B). It is obvious that the curves fall at an ever increasing rate; *i.e.*, faster than would be expected for a population or populations being intoxicated in accordance with first-order kinetics. These results appear to suggest a normal distribution of intoxication in time, provided the lag period is excluded from consideration. Probit analysis also indicates such a distribution.

Addition of S^{35}-Methionine to Fully Intoxicated Cells.—It can be seen from Fig. 4 A that by 1.5 hours the intoxication in the presence of saturating levels of toxin is complete, but the incorporation of methionine continues at a declining rate for another hour. The possibility that such fully intoxicated cell suspensions were impermeable to methionine was tested for by the addition of

antitoxin (as a control) and S^{35}-methionine 1.5 hours after the addition of toxin. The kinetics of incorporation were identical with the intoxicated control (Fig. 6), indicating that intoxication exerts no effect on the entrance of S^{35}-methionine into the cell.

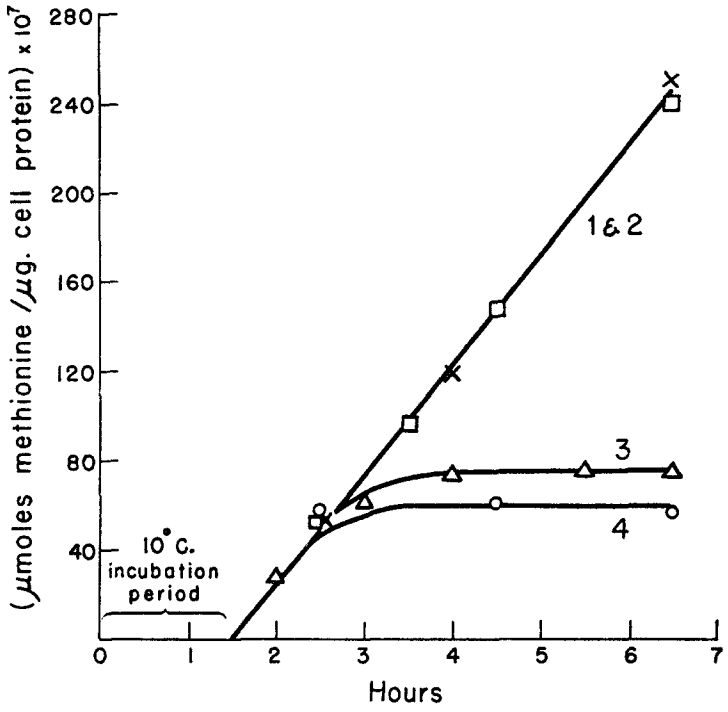

FIG. 7. The effect of low temperature on the intoxication of cells.

(1) Cells kept at 10°C. during the 1.5 hours' incubation. S^{35}-methionine added at 1.5 hours.

(2) Cells chilled to 10°C. and toxin added at zero time. Cells incubated for 1.5 hours at 10°C. Antitoxin and S^{35}-methionine added and cells warmed to 37°C.

(3) Cells chilled to 10°C. and toxin added at zero time. After 1.5 hours, S^{35}-methionine was added and the cells warmed to 37°C.

(4) Cells chilled to 10°C. for 1.5 hours. Toxin and S^{35}-methionine added.

All toxin was added to a final concentration of 0.3 Lf per ml.

The Effect of Temperature on Intoxications.—The incubation of cells with toxin at 10°C. for 1.5 hours in the presence of saturating levels of toxin revealed that toxin fails to intoxicate at this low temperature.

A cell suspension was prepared in the usual manner, was divided into four parts, and chilled to 10°C. Two of the flasks received toxin to a final concentration of 0.3 Lf per ml. All four flasks were then incubated at 10°C. for 1.5 hours. At the end of this period all four flasks

received S^{25}-methionine. One of the toxin-containing flasks received excess antitoxin, and one of the control flasks now received toxin to a level of 0.3 Lf per ml. All four flasks were then quickly warmed to 37°C. and samples taken at intervals for measurement of S^{35}-methionine incorporated. The results are shown in Fig. 7.

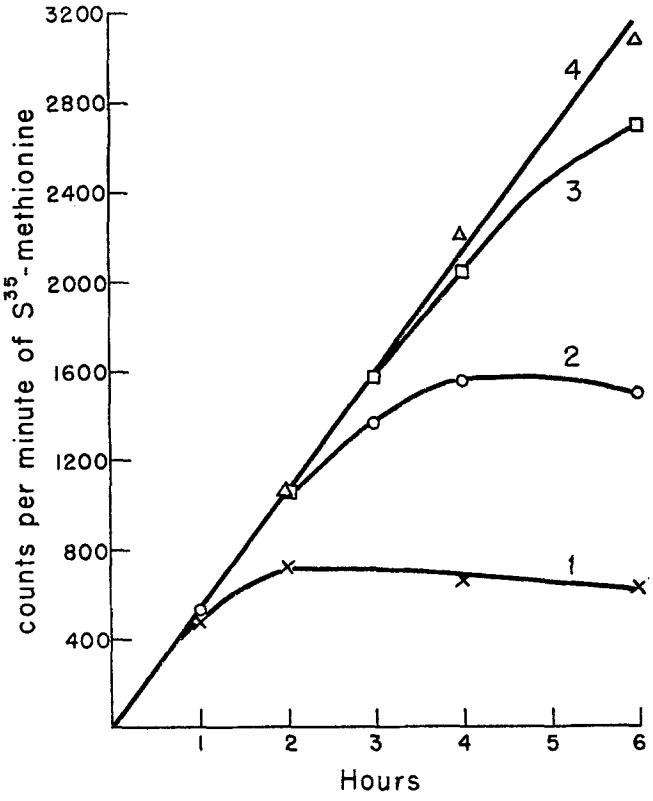

FIG. 8. The adsorption of toxin by HeLa cells.

(1) Cells exposed to toxin for 10 minutes and chilled to 10°C.

(2) Cells exposed to toxin for 10 minutes, chilled to 10°C., centrifuged, and resuspended in complete medium.

(3) Cells exposed to toxin for 10 minutes, chilled to 10°C., washed once by centrifugation, and resuspended in complete medium.

(4) Non-intoxicated control chilled to 10°C.

Following completion of washing of flasks 2 and 3, all flasks were warmed to 37°C. and S^{35}-methionine added. Toxin used as 0.3 Lf per ml. final concentration.

It is clear that (1) the chilling had no effect on the subsequent ability of the cell to incorporate S^{35}-methionine: (2) toxin fails to intoxicate at low temperatures; (3) antitoxin acts rapidly to neutralize toxin in the suspension under these conditions. To eliminate the possibility that cells protected with anti-

toxin after incubation with toxin in the cold were actually intoxicated but failed to show this intoxication in short term experiments, cells proliferating on a glass surface were exposed to toxin for 1.5 hours at 10°C. After adding antitoxin they were warmed to 37°C. and observed at intervals microscopically for several days. They showed no evidence of intoxication.

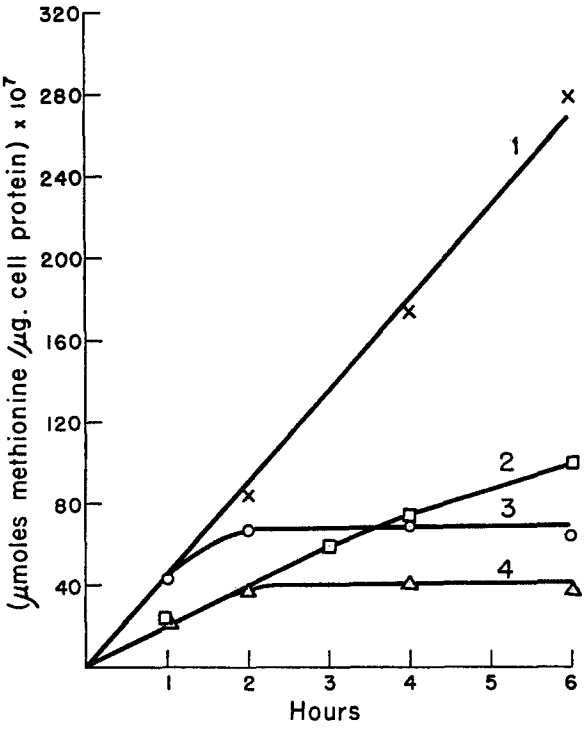

FIG. 9. A comparison of the effect of cyanide with that of toxin on HeLa cells.
(1) Non-intoxicated control.
(2) Cyanide alone.
(3) Toxin alone.
(4) Cyanide and toxin.
Cyanide concentration 0.001 M. Toxin concentration was 0.3 Lf per ml.

The Adsorption[4] *of Toxin.*—It had previously been found that saturating concentrations of toxin, when incubated with cells for 10 minutes at 37°C. failed to cause any observable inhibition of protein synthesis (Fig. 4 A). Using this information, and that obtained in the previous experiment, an examination of the adsorption of toxin by the cell was undertaken.

[4] At this point it is worth noting that although the term "adsorption" is used, it is not known whether this attachment of toxin to the cell is superficial, or whether toxin is absorbed by the cells.

Toxin was allowed to act on cells at saturating concentrations for 10 minutes at 37°C. The cells were quickly chilled to 10°C., centrifuged, and either resuspended in fresh medium, or washed once and then resuspended. The cells were warmed to 37°C. and S^{35}-methionine added. Aliquots of suspension were removed at intervals for the determination of radioactivity.

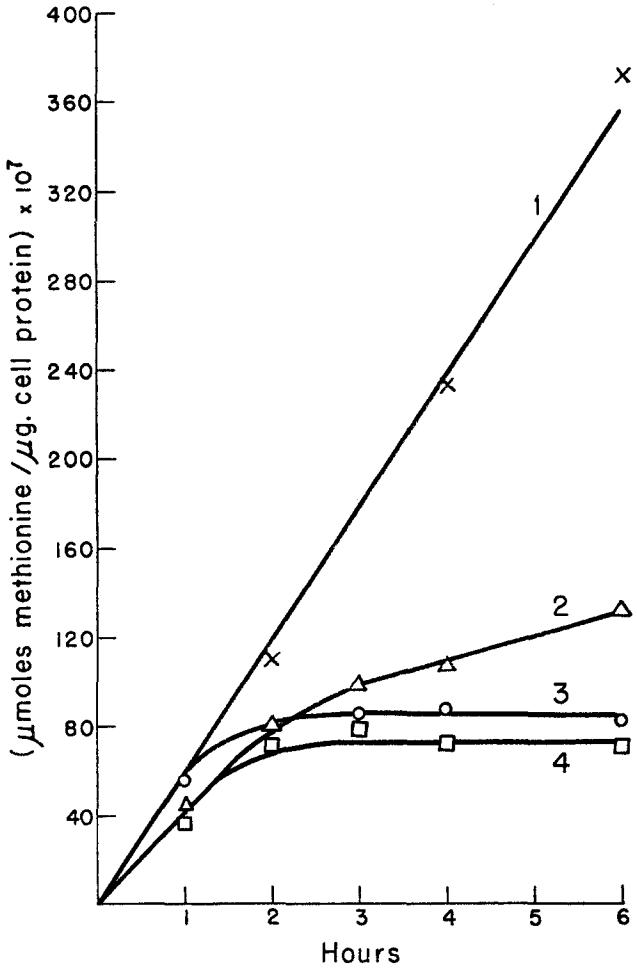

FIG. 10. A comparison of the effect of 2,4-dinitrophenol with that of diphtheria toxin on HeLa cells.

1. Non-intoxicated control.
2. 2,4-dinitrophenol alone.
3. Toxin alone.
4. 2,4-dinitrophenol and toxin.

Dinitrophenol concentration 0.003 M. Toxin concentration 0.3 Lf per ml.

As can be seen from Fig. 8, the period of normal incorporation increased with each washing. However, the washing corresponded to at least a 1000-fold dilution of the original toxin, yet the cells behaved as if the original toxin were only 30 times as dilute. These results indicate that toxin is adsorbed rapidly and released slowly with each washing.

The Effect of Cyanide and 2,4-Dinitrophenol.—It has been suggested (8) that a relationship exists between succinoxidase activity and sensitivity to diphtheria toxin. It was of interest, therefore, to compare the effect on methionine uptake of certain agents which affect respiratory systems, to that of diphtheria toxin. In Figs. 9 and 10 can be seen the effect on S^{35}-methionine incorporation of 10^{-3} M cyanide and 3×10^{-3} M 2,4-DNP, both in the presence and absence of toxin. It can be seen that both of these reagents when used alone, exert their effects instantaneously as evidenced by a slower initial rate of methionine uptake. Moreover, these reagents do not affect the kinetics of intoxication when used in the presence of toxin. Apart from a comparison of the kinetics of toxin action with that of the respiratory inhibitors, it is of interest that toxin eliminates *completely* any methionine uptake remaining after the addition of cyanide or 2,4 DNP.

Cells treated with these concentrations of cyanide or 2,4-DNP in the absence of toxin appear microscopically normal for many hours in contrast to the action of saturating toxin concentrations, which cause visible cell damage within 4 to 7 hours.

DISCUSSION

The information obtained in the present study concerning the kinetics of action of diphtheria toxin and its effect on certain metabolic systems permits a narrowing down of the many possibilities which might conceivably offer an explanation for its mode of action. It can be concluded from this work that glycolysis and aerobic respiration are not acted on directly by toxin. Protein synthesis was shown to be completely inhibited 2 hours after the administration of saturating concentrations of toxin. This early suppression of methionine incorporation is all the more striking when one considers that morphological changes cannot be observed at least until the 4th hour.

The data obtained from radioisotope experiments suggest that the period of normal methionine uptake following the addition of toxin to a cell suspension can be attributed to several factors. A minor part of this "lag" must be the time necessary for the adsorption of toxin by the cells. This is evident from experiments in which cells had been exposed to toxin for 10 minutes at 37°C., chilled to prevent further intoxication, washed once or twice, and then allowed to incorporate S^{35}-methionine at 37°C. (Fig. 8). The resulting kinetics of incorporation suggest a rapid adsorption of toxin by the cell. It is clear that this

adsorption is quite distinct from the process by which toxin causes irreversible damage. This fact is indicated both by the reversibility of the adsorption, and by the observation that such adsorbed toxin can be neutralized almost instantaneously by antitoxin so that no measurable cell damage ensues. These results are in agreement with those of Placido Sousa and Evans (10).

At this point, it is worthwhile commenting on the significance of this reversible adsorption of toxin with respect to variations in resistance to toxin. Mammalian cell lines derived from toxin-sensitive animals show extreme variability in their susceptibility to toxin. Lennox and Kaplan (9) reported that rabbit kidney cells were ten times more sensitive than HeLa, and that altered monkey kidney cells (25) were resistant to toxin whereas normal monkey kidney cells were sensitive. These examples serve to emphasize the wide range of sensitivity which exists among various cell types. It is reasonable to assume that such a wide range of susceptibility also exists between the cells of different tissues *in vivo*. A variety of factors may serve to explain these differences. Absence of the specific target, decreased permeability to toxin, or a decreased ability to adsorb toxin on the cell surface are the most obvious possibilities. Of the first two possibilities we know little. The latter possibility should be considered in view of the primary adsorption of toxin which we have observed in the case of the HeLa cell. Whether or not this adsorption is specific for toxin is not known. Many proteins can be non-specifically adsorbed by cells and washed off only with difficulty. However, regardless of whether or not the adsorption is specific, it is obvious that the rate of intoxication is dependent on the amount adsorbed. Variations in the surface structure affecting the affinity for toxin might therefore be expected to affect the number of toxin molecules per unit of cell surface at equilibrium. Such variations are certainly present among the cells of various tissues in susceptible animals and might serve to explain, at least in part, the increase in sensitivity which chicken embryo heart tissue undergoes at about the thirteenth day of its development (26, 27) as well as the refractoriness of rat and mouse tissues to toxin.

Concerning the major portion of the observed period of normal uptake of methionine by intoxicated cells little information has been obtained. This period may be attributed to one or a combination of several phenomena: such as the time necessary for the accumulation of a destructive substance, the exhaustion of a vital pool of material, or the destruction of some cellular structure which directly affects protein synthesis. Little can be said at present concerning these possibilities. In any event, it is clear that concentrations of toxin which are minute from a chemical standpoint can rapidly exert an extremely destructive effect on cells.[5] In this respect it is interesting to compare the effects of

[5] Assuming 1 Lf to contain 2.4 micrograms of toxic protein (29), that toxin has a molecular weight of 70,000 (30), and that every molecule is biologically active, it can be calculated that a saturating level of 0.3 Lf per ml. is 10^{-8} M in toxin, although a culture can be completely destroyed by 0.05 Lf per ml. or less.

relatively benign toxic materials such as cyanide and 2,4-DNP. Concentrations of these chemicals some 100,000 times greater than the concentration of diphtheria toxin still permit the cells to retain an apparently normal appearance for many hours.

The metabolic level at which diphtheria toxin interferes with protein synthesis remains to be determined. Interference with the ability of the cell to form or to utilize high energy compounds, specific elimination of ribonucleic acid synthesis, or a direct attack on protein synthesis could account for the failure of the intoxicated cell to lay down methionine.

The status of a partially intoxicated population is a problem the solution to which is of considerable importance to an understanding of the mechanism of intoxication. As suggested by experiments in which antitoxin was added to a cell suspension during the "lag" period (Figs. 4 A to 4 C, 5 A to 5 B), the suspension loses the ability to incorporate methionine as a function of time in accordance with a normal distribution. Unfortunately, it is not possible to decide on the basis of these data whether reduced methionine uptake by a partially intoxicated culture is a measure of surviving non-intoxicated cells, or is a measure of impairment of protein synthesis by every cell.

The observation that 0.001 M cyanide and 0.003 M DNP fail to completely eliminate methionine incorporation would appear to be in accord with the observed high glycolytic rate of these cells as well as the recent report that HeLa cells can synthesize polio virus under anaerobic conditions (28). In contrast, toxin *completely* eliminates methionine incorporation, whether used in the presence or absence of cyanide or DNP. If the residual methionine uptake occurring in the presence of cyanide or DNP is indeed due to energy derived from the glycolytic breakdown of glucose, then it would appear that diphtheria toxin interferes with the utilization of this energy for the synthesis of protein. Were the action of toxin that of specifically uncoupling phosphorylation from oxidation, one might expect, even in its presence, some remaining methionine incorporation as a result of energy provided by the glycolytic process. Such apparently is not the case. This finding thus failed to enlarge upon the striking relationship between the succindehydrogenase system and sensitivity to toxin demonstrated in the metamorphosing *cecropia* silkworm (8).

SUMMARY

The effect of diphtheria toxin on certain metabolic processes in growing HeLa cells has been described. It was found that both aerobic respiration and glycolysis are insensitive to toxin action. The incorporation of S^{35}-methionine was found to be completely inhibited by toxin prior to the appearance of any visible cell damage. It was also observed that toxin levels above 0.3 Lf per ml. had no further effect on the kinetics of inhibition of methionine uptake. The kinetics of intoxication were studied by adding diphtheria antitoxin to a

partially intoxicated culture at various times and measuring the resulting rate of methionine uptake.

Low temperatures were found to block intoxication. Using this information, data were obtained suggesting that the initial step in the process of intoxication consists of a rapid adsorption of toxin by the cell.

The kinetics of toxin action on protein synthesis were compared with those of cyanide and 2,4-DNP. Diphtheria toxin eliminates completely any methionine uptake remaining after exposure of the cell to either of these respiratory inhibitors.

The significance of these results is discussed.

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