THE EFFECT OF DIPHTHERIA TOXIN ON THE METABOLISM OF HeLa CELLS

II. Effect on Nucleic Acid Metabolism*, ‡

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Previous studies (1) on the mechanism of action of diphtheria toxin have shown that protein synthesis in a mammalian cell culture (HeLa), as measured by S^{35}-methionine incorporation, is inhibited completely in the presence of saturating concentrations (0.3 L_f units per ml.) of a purified toxin. This inhibition is extremely rapid on a physiological time scale, complete cessation of protein synthesis by the HeLa cell occurring in 1 to 2 hours.[1] The time course of S^{35}-methionine incorporation in the presence of saturating toxin levels is characterized by an initial 1 hour period of normal incorporation followed by a complete cessation in uptake. The kinetics of this inhibition have been characterized with regard to effect of antitoxin, effect of temperature, and the effect of dinitrophenol and cyanide. Pappenheimer and Williams (2, 3) have postulated, on the basis of evidence obtained with the silkworm *Platysamia cecropia*, that toxin was either preventing cytochrome synthesis or uncoupling oxidation from phosphorylation. The first of these possibilities has been eliminated definitely by the finding that all protein synthesis is blocked by toxin in considerably less than one generation time. The second possibility remains to be tested. With this in mind, an examination of the effect of toxin on other biosynthetic pathways was undertaken to ascertain whether or not a general cessation of synthetic processes prevails in the presence of toxin.

The work to be described is concerned with the effect of diphtheria toxin on the synthesis of cell components other than protein, specifically nucleic acids, and the acid-soluble organic phosphates.

Materials and Methods

Suspensions of HeLa, strain S-3 (4, 5) were prepared as described previously (1). The cells were washed twice in Eagle's medium (6) containing 10 per cent horse serum (BME-10) and

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[1] The HeLa cell under these conditions has a generation time of about 24 hours.

suspended in Eagle's medium containing 1 per cent horse serum (BME-1) to a level of about 10^5 cells per ml. The flasks were then placed in a 37°C. "gyrotary" water bath and allowed to equilibrate for 15 minutes prior to the addition of toxin or other reagents. Measurements of thymidine, uridine, and S^{35}-methionine incorporation were made as previously described (1).

For experiments involving the incorporation of inorganic P^{32} the cells were grown in Roux bottles, treated with 0.005 M EDTA to remove them from the glass, washed twice in medium BME-10 and once in medium BME-1. The cells were suspended to a level of 1 to 2 × 10^6 cells per ml. in BME-1. The suspensions were placed in a 37°C. water bath and allowed to equilibrate for 20 minutes prior to addition of reagents. Aliquots were removed at various intervals after time zero, and transferred directly to 5 per cent trichloracetic acid (TCA) containing 0.02 M non-radioactive phosphate. This served to eliminate virtually all non-specific P^{32} orthophosphate adsorption to the charcoal in the ensuing step, in which aliquots of the TCA supernatant were treated with Norit A to adsorb nucleoside phosphates. To determine acid-labile phosphate the Norit-adsorbed material was washed thoroughly and treated with 1 N HCl at 100°C. for 10 minutes (7). The supernatant was assayed for P^{32}. Nucleic acid phosphorus was determined by assay of the hot TCA-soluble fraction for P^{32}.

For experiments involving leakage of P^{32}, cells were grown on glass in the presence of P^{32} orthophosphate (2 × 10^7 C.P.M. per ml.) for 72 hours. The radioactive medium was removed, cells washed with complete medium several times and covered with complete medium. Toxin was added at zero time to a level of 1 L_f per ml. and 0.1 ml. aliquots of the supernatant removed and plated for P^{32} determinations.

Chemicals.—Tritiated thymidine having a specific activity of 2.7 curies per millimole, tritiated uridine having a specific activity of 0.62 curies per millimole and S^{35}-methionine were obtained from Schwarz Laboratories, Inc., Mt. Vernon, New York. Carrier-free P^{32} orthophosphate was obtained from the Oak Ridge Laboratories in Tennessee.

Diphtheria Toxin[2].—Toxin was prepared according to the method of Yoneda (8). The toxin contains 1500 L_f per ml. and about 40 guinea pig M.L.D. per L_f.

Measurements of Radioactivity.—In all cases, samples were plated at infinite thinness and counted by means of a Nuclear-Chicago windowless gas-flow counter.

RESULTS

Effect of Toxin on Nucleic Acid Synthesis.—As can be seen from Fig. 1, the incorporation of tritiated thymidine into acid-insoluble material is less sensitive to the presence of saturating concentrations (1) of diphtheria toxin than is S^{35}-methionine incorporation. Similarly, the incorporation of uridine (Fig. 2) is resistant to the effect of toxin. Incorporation of uridine by the intoxicated cells proceeds normally until the 3rd to 4th hour, at which time a marked decrease in rate becomes noticeable.

The incorporation of both thymidine and uridine are immediately sensitive to 10^{-3} M 2,4-dinitrophenol to the extent of about 80 per cent inhibition of the rate of incorporation.

These results indicate that a general uncoupling of oxidation from phosphorylation by toxin is not a likely explanation of the lethal effect of toxin, inasmuch as the extent of inhibition of nucleic acid synthesis is considerably

[2] Some samples of crude toxin were kindly supplied by the Biologic Laboratories, Boston, Massachusetts Department of Public Health.

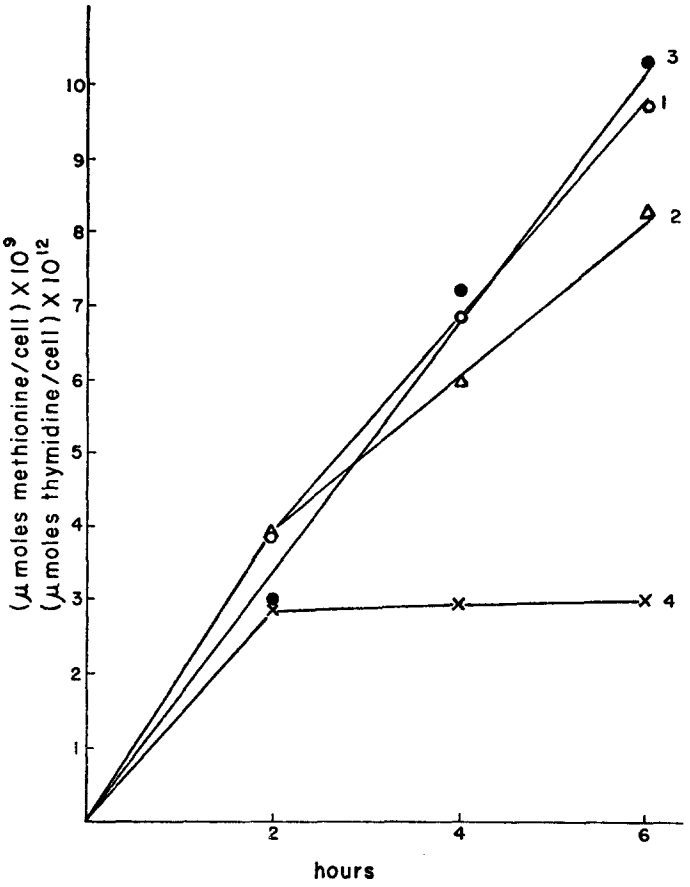

FIG. 1. The incorporation of tritiated thymidine into DNA. Four-day old cells growing on glass were versenized, washed, suspended in BME-1 to a level of 1.1 × 10^5 per ml., and equilibrated 20 minutes at 37°C. in a rotatory water bath. At zero time tritiated thymidine and toxin were added. Final thymidine concentration was 200,000 C.P.M. per ml. Final toxin concentration was 0.3 L_f per ml. S^{35}-methionine incorporation was carried out in parallel in order to compare the kinetics.

Curve 1, thymidine; Curve 2, thymidine plus toxin; Curve 3, S^{35}-methionine; Curve 4, S^{35}-methionine plus toxin.

less than was obtained with dinitrophenol. The possibility was considered that energy for the synthesis of nucleic acids in the presence of toxin was obtained from pre-existing pools of nucleotides. Consequently, an examination of the effect of toxin on the synthesis of these compounds was carried out.

Effect of Toxin on Acid-soluble Components.—The incorporation of P^{32} into the cold acid-soluble, charcoal-adsorbable fraction (nucleoside phosphates) is only slightly susceptible to the action of toxin (Fig. 3) in the observed time

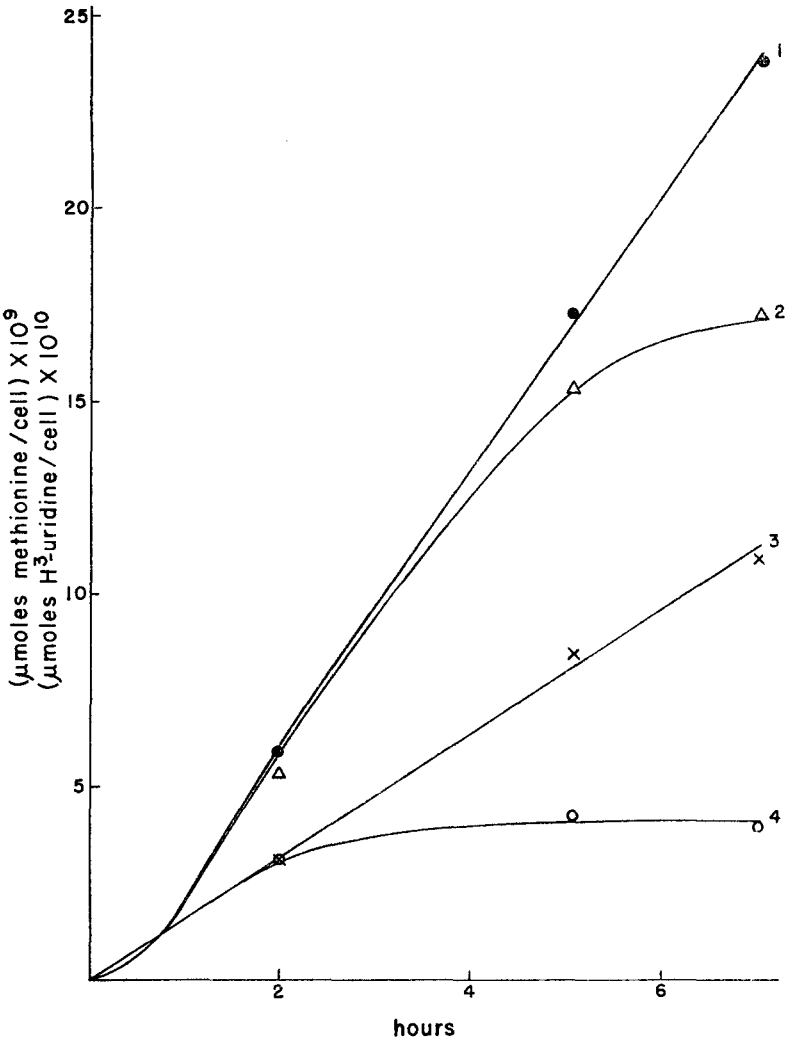

FIG. 2. The incorporation of tritiated uridine into RNA. Conditions as per Fig. 1, but uridine added instead of thymidine in a final concentration of 190,000 C.P.M. per ml.

Curve 1, uridine; Curve 2, uridine plus toxin; Curve 3, S^{35}-methionine; Curve 4, S^{35}-methionine plus toxin.

interval of 2 hours, when toxin and P^{32} are added simultaneously. This experiment was also carried out by preincubating the cells with toxin for 15 minutes and then adding P^{32}. Here, again, no effect is evident on the rate of P^{32} incorporation into this fraction. It can be seen from Table I that an extremely high toxin concentration causes only 6 per cent inhibition of the rate of P^{32} incor-

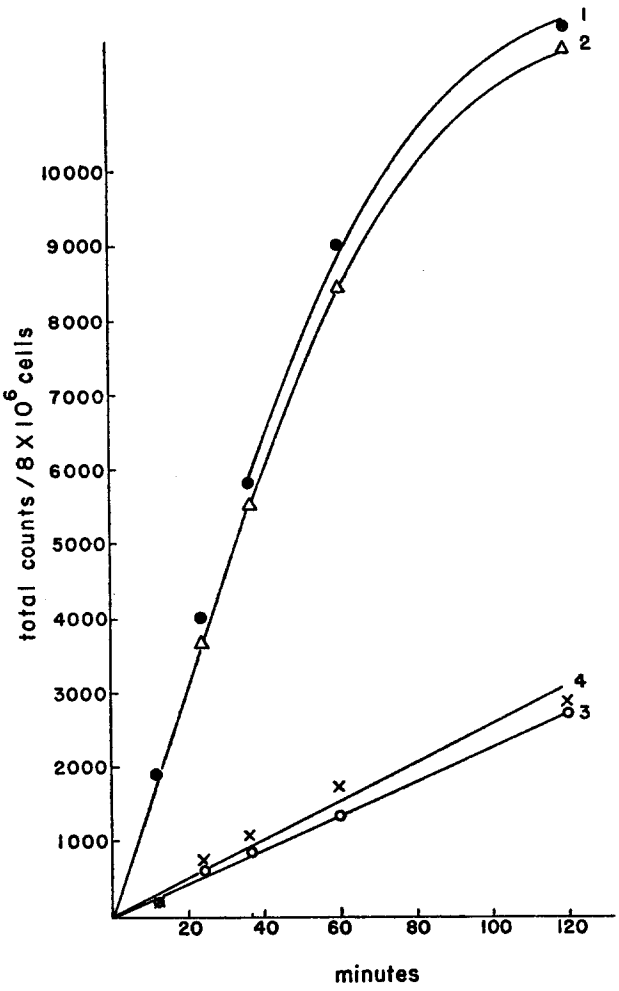

FIG. 3. Incorporation of P^{32} into the nucleoside phosphate and nucleic acid fractions. Cells were suspended as described in the text. At zero time, carrier-free P^{32} orthophosphate was added to give a final concentration of 2.7×10^6 C.P.M. per ml.

Curve 1, control, nucleoside phosphates; Curve 2, intoxicated, nucleoside phosphates; Curve 3, control, nucleic acid; Curve 4, intoxicated, nucleic acid.

poration as compared to the control. The incorporation is sensitive, however, to the action of dinitrophenol (5×10^{-4}M) to the extent of 40 to 50 per cent inhibition (Table I).

It can also be seen from Fig. 3 that the incorporation of P^{32} into the charcoal-adsorbable fraction continues at a linear rate for some 60 minutes. In one experiment it was found, on the basis of radioactivity, that during this period

TABLE I

Effect of Dinitrophenol and Toxin on P^{32} Incorporation into Nucleoside Phosphates

Addition	Final concentration	P^{32} incorporation at: (μ mole per cell) $\times 10^{11}$		Per cent inhibition of rate
		30 min.	*60 min.*	
None	—	7.9	17.2	—
Toxin	22.5L_f per ml.	7.4	16.1	6.4
2,4-DNP	5×10^{-4} M	4.1	9.0	48.0

Conditions as for Fig. 3.

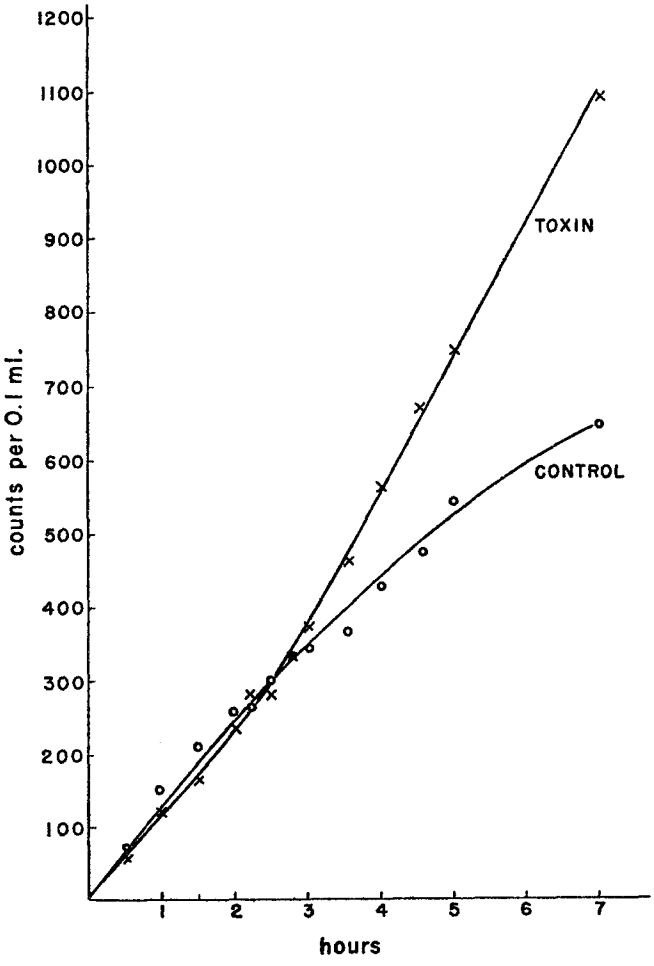

FIG. 4. P^{32} leakage from intoxicated cells. This experiment was carried out as described in the text.

of linear uptake only 1.5×10^{-3} micromoles of phosphorus per 2×10^6 cells had been incorporated into the charcoal-adsorbable fraction during a 25 minute interval, whereas phosphate determinations during this same period showed 0.65 micromoles phosphorus taken up. This indicates that during this period of linear uptake there is a net synthesis of acid-soluble organic phosphates, and that the steady state has not yet been attained.

The above experiment is compatible with the observation (Fig. 4) that cells grown in P^{32} and treated with toxin exhibit no leakage of P^{32} in excess of the control prior to 1.5 hours, at which time accelerated release of P^{32} commences, this coinciding with cessation of methionine incorporation. This indicates that very little cell membrane damage has occurred prior to 1 hour. The nature of the P^{32} which leaks from the cell after 2 hours in the presence of toxin is not known.

Effect of Increased Amino Acid Concentration.—To test the possibility that protein synthesis ceases because of leakage of amino acid pools, the intoxication was carried out in the presence of three times the normal concentration of amino acids. The kinetics of intoxication as measured by S^{35}-methionine incorporation showed no measurable differences from those exhibited in the presence of the usual concentrations of amino acids. Although this result does not rule out cell membrane damage, it does suggest that amino acid leakage *per se* is not responsible for the rapid cessation of protein synthesis.

DISCUSSION

The suggestion that toxin acts solely by uncoupling oxidation from phosphorylation (3) does not seem likely on the basis of available evidence: Toxin completely inhibits the incorporation of methionine into protein in 1 to 2 hours, whereas known uncouplers such as cyanide and DNP fail, at high concentrations, to inhibit completely such incorporation. Toxin fails to affect nucleic acid synthesis to any great degree until 3 to 4 hours have elapsed, whereas DNP (10^{-3} M) causes an immediate 80 per cent loss in the rate of synthesis. Finally, toxin fails to block the incorporation of P^{32} into the acid-soluble fraction, whereas DNP inhibits this incorporation some 40 per cent. Thus, not only does toxin fail to follow the pattern of known uncouplers, but its effects on the cell are of such a drastic nature as to cause cell disruption starting at 4 to 5 hours (1).

The results described above also serve to characterize further the action of diphtheria toxin on the HeLa cell. Of the several metabolic processes observed during intoxication, protein synthesis shows the most sensitivity to toxin. Either of two general mechanisms may be postulated to explain the action of toxin: (*a*) toxin acts specifically on protein synthesis, resulting in secondary effects which eventually derange nucleic acid synthesis and cause extensive cell degeneration; or (*b*) toxin primarily affects a cell structure, such as the cell membrane, this in turn causing secondary effects which culminate in a

cessation of all cell processes. The latter possibility seems at present to be most plausible, although the first possibility has by no means been eliminated from consideration.

The strongest evidence in favor of cell membrane damage as the mechanism of toxin action is the rapidity with which the cells begin to disintegrate (4 hours) (1) and the simultaneous cessation of protein synthesis and acceleration of P^{32} leakage at 1 to 2 hours. Moreover, the very nature of the toxin molecule makes it improbable that it would enter the cell. It is of molecular weight 70,000 (9) and the pH at the isoelectric point is 4.5 (10). These two properties alone make rapid diffusion unlikely. The possibility that any form of active transport may be involved (pinocytosis) in the uptake of toxin is made improbable by the observation that destruction of the cell by diphtheria toxin can occur in the presence of high concentrations of 2,4-dinitrophenol with exactly the same kinetics as are found when toxin acts alone (1). That cell membrane damage has not occurred prior to 1 to 2 hours after addition of toxin is indicated by: (*a*) failure of high exogenous amino acid concentrations to alter the course of intoxication; (*b*) normal incorporation of P^{32} orthophosphate into acid-soluble organic components for at least 2 hours; (*c*) normal leakage rate of P^{32} from prelabeled cells for 1 to 2 hours.

None of the pieces of evidence listed above are conclusive enough either singly or taken as a whole to warrant acceptance of either mechanism. Definitive experiments are now in progress to test further these two possible mechanisms.

Addendum.—During the writing of this manuscript, Kato and Pappenheimer (11) kindly sent us a copy of their completed manuscript concerning studies on the effects of diphtheria toxin on both HeLa and human kidney cell cultures. They have confirmed and extended the earlier work of this author (1). They obtained essentially the same results in respect to the effects of diphtheria toxin on nucleic acid synthesis as are described in this paper. In addition, they have demonstrated in an elegant series of experiments an instantaneous 40 per cent inhibition by diphtheria toxin of P^{32} incorporation into the nucleoside phosphate fraction under steady state conditions. This inhibition was shown to be immediately reversible by antitoxin up to 30 minutes after the addition of toxin.

These results appear to be at variance with the results described in this paper (Fig. 3). A possible explanation is that under the conditions prevailing in our experiments the cells are not in the steady state. This may mask a 40 per cent inhibition of incorporation of P^{32} into nucleotides formed at the membrane.

The crucial consideration is whether this effect of toxin on ATP synthesis *per se* is responsible for cell death, or whether this effect is due to the attack by toxin on the cell membrane at precisely those sites responsible for ATP synthesis.

SUMMARY

The effect of toxin on the incorporation of uridine and thymidine into nucleic acids was investigated. P^{32} orthophosphate incorporation into the

nucleoside phosphate fraction was also investigated. No early effect of toxin on nucleic acid synthesis was evident and only a slight effect of toxin on nucleoside phosphate formation was found prior to 1.5 hours after toxin addition. Both these processes, however, were found to be sensitive to dinitrophenol. These results are interpreted as indicating that toxin does not act by a general uncoupling of oxidation from phosphorylation. Other possible mechanisms of toxin action are proposed.

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