AN EARLY EFFECT OF DIPHTHERIA TOXIN ON THE METABOLISM OF MAMMALIAN CELLS GROWING IN CULTURE*

By IWAO KATO,‡ M.D., and A. M. PAPPENHEIMER, Jr., Ph.D.

(From The Biological Laboratories, Harvard University, Cambridge)

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Strauss and Hendee (1) recently studied the effect of purified diphtheria toxin on the metabolism of HeLa cells. They followed protein synthesis in suspensions of cells growing in culture by measuring the kinetics of incorporation of S^{35}-methionine into cell protein over periods of 5 to 6 hours. They found that, after addition of *saturating* doses of toxin (1 μg. or more per ml.), growth and protein synthesis at 37°C. proceed at their normal linear rate for a period of 75 to 90 minutes at which time the rate of methionine uptake decreases sharply and, within 2 hours of adding the toxin, ceases altogether. Morphological damage cannot be observed until at least the 4th or 5th hour and respiration remains unaffected in the presence of toxin for even longer periods. Strauss and Hendee found that the inhibition of S^{35}-methionine incorporation into cell protein by saturating doses of toxin could be prevented almost completely by addition of excess diphtheria antitoxin within 30 minutes after adding toxin and, even when added 60 minutes after toxin, S^{35}-methionine incorporation into protein continued at about one-half the initial rate for so long as it was measured. With smaller doses of toxin, the latent period of normal growth and methionine incorporation was prolonged and inhibition could be prevented to an appreciable extent by antitoxin up to 3 hours after addition of toxin.

In the present studies, we have confirmed and extended these striking experiments of Strauss and Hendee using the MK strain of normal human kidney cells. In addition we have investigated the incorporation of inorganic radiophosphorus (P_i^{32}) into nucleic acids and into adenine nucleotides. We have been able to demonstrate that saturating doses of purified diphtheria toxin cause a striking and instantaneous inhibition of P_i^{32} incorporation into adenosinetriphosphate (ATP) by growing HeLa and human kidney (MK) cells.

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Materials and Methods

Cell Lines.—Only cell lines that had become established in tissue culture were used. We are indebted to Dr. John F. Enders for the culture of HeLa cells and for a culture of normal human kidney cells MK. Dr. George E. Foley kindly supplied us with a culture of mouse spleen cells CCRF-222, isolated from a normal CFW mouse and carried through 37 transfers in his laboratory (2).

Culture Media.—Cell lines were maintained at 37°C. in Eagle's medium (3) containing 10 per cent calf serum in the case of HeLa and human kidney (MK) cells or 5 per cent calf serum in the case of mouse spleen cells. Every 24 or 48 hours, the medium was decanted and replaced with fresh medium. Cells were removed from the glass surface with trypsin, centrifuged, washed with Hanks' solution, and reinoculated into fresh medium about every 4th day.

Cells used in the experiments to be described were heavily inoculated, approximately 10^5 cells per ml., into Roux bottles containing 50 ml. of medium. 48 hours later, the cells were removed from the glass with 0.005 M versene, rather than trypsin. They were centrifuged, washed 3 times with Hanks' solution and suspended in Eagle's medium specifically modified according to the nature of the experiment to be performed. The medium contained 2 per cent calf serum that had previously been dialyzed with stirring in the cold against changes of saline in order to remove free amino acids, glucose, etc.

Measurement of Cell Density.—Cells were either counted directly according to the method of Sanford *et al.* (4) or were measured in terms of total cell protein. Protein was determined on aliquots of washed cell suspensions by the method of Lowry *et al.* (5) as modified by Oyama and Eagle (6). Crystalline bovine serum albumin (Armour) was used as a standard. 150 μg. cell protein were found to be equivalent to approximately 2×10^5 cells. In most experiments cell densities were adjusted to 100 to 150 μg. protein per ml.

Diphtheria Toxin.—Toxin was prepared and purified by the method of Yoneda (7). The stock solution was kept in the frozen state and contained 4500 Lf/ml. and 75 M.L.D./Lf. In most of the experiments to be described, toxin was used at a concentration of 1 Lf/ml. ($\simeq$2.5 μg./ml.).

Labeled Compounds.—S^{35}-L-methionine (1000 to 2500 counts per μg. depending upon lot number) was obtained from Schwarz Laboratories, Mount Vernon, New York. It was purified by chromatographing on paper using butanol–acetic acid–water as solvent. 6-C^{14}-orotic acid from New England Nuclear Corp., Boston, (2840 counts per μg.) was used without further purification. Carrier-free $H_3P^{32}O_4$ from Oak Ridge Laboratories, Tennessee, was used as the source of inorganic phosphate. We are grateful to Dr. A. K. Solomon for making potassium-42 (Brookhaven, National Laboratory, Upton, Long Island) available to us.

S^{35}-methionine incorporation was followed as described by Strauss and Hendee (1). Cell suspensions were prepared in Eagle's medium containing 2 per cent dialyzed calf serum and lacking methionine. 10 μg. S^{35}-methionine per ml. were added at the start of each experiment At suitable intervals, 1 ml. samples were collected on Millipore filters, and washed, first with cold Hanks' solution (saline A) and then with chilled 5 per cent trichloroacetic acid (TCA) containing 150 μg. non-radioactive methionine per ml. The filters were mounted on copper discs, dried, and counted in an automatic thin window gas flow counter.

Incorporation of P_i^{32} into RNA and DNA.—Cells grown for 48 hours in Roux bottles were collected, centrifuged, and washed in the usual manner and then resuspended in Eagle's medium containing 2 per cent dialyzed calf serum in two 300 ml. Erlenmeyer flasks (60 ml. per flask) to a cell density of about 125 μg. protein per ml. To each flask was added 120 μc. carrier-free P_i^{32}. Diphtheria toxin to a concentration of 1 Lf/ml. was added to one of the flasks. The flasks were rotated in the water bath at 37°C. and 20 ml. aliquots were removed for analysis after 1, 2, and 3 hours' incubation. The samples were centrifuged in the cold, washed

3 times with cold Hanks' solution (saline A), and each washed residue, containing *ca.* 2.5 mg. cell protein, was suspended in 1 ml. Hanks' solution mixed with 2.5 ml. cold 10 per cent TCA, and centrifuged. The residue was resuspended in 2.5 ml. 10 per cent TCA, recentrifuged, and the ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) were separated according to the methods of Schmidt and Thannhauser (8) and Schneider (9) as follows. The TCA residue was suspended in 1 ml. distilled water and the nucleic acids were precipitated with 4 ml. ethanol. After centrifuging, the supernate was discarded and the precipitate washed with 5 ml. ethanol in the centrifuge. It was then extracted 3 times at room temperature with alcohol-ether (3:1). The final residue was collected by centrifugation and treated for 18 hours at 37°C. with 1 ml. 1 N KOH after which the turbid solution was neutralized with 5 per cent perchloric acid and centrifuged. The neutral supernate containing the RNA fraction was treated with 1 ml. 2.5 per cent $CaCl_2 \cdot 2H_2O$ to precipitate inorganic phosphate. 1 ml. aliquots of the P_i-free RNA fraction made up to 4 ml. were analyzed for total phosphorus by the Dryer *et al.* (10) modification of the Fiske and Subbarow (11) method and other aliquots were dried on planchets and counted for P^{32}. Results were expressed as P^{32} counts per microgram RNA-phosphorus.

The perchloric acid–insoluble DNA-containing fraction was resuspended in 5 ml. of 5 per cent $HClO_4$, heated 15 minutes at 90°C., cooled, and centrifuged. The combined extracts made up to 10 ml. were analyzed for phosphorus as above and their P^{32} content determined.

Incorporation of P_i^{32} into Adenine Nucleotide Fraction.—Growing cell suspensions (150 to 200 μg. cell protein per ml.) in Eagle's medium containing 2 per cent dialyzed calf serum were dispensed into 50 ml. flasks in 10 ml. portions. After 30 minutes in the rotating water bath at 37°C., 100 μc. P_i^{32} were added per flask. After exactly 2 minutes' further shaking, the contents of each flask were rapidly chilled to 5–10°C., centrifuged in the cold, and washed 3 times with chilled Hanks' solution (saline A). The cell residue was extracted with 5 ml. cold 10 per cent TCA and the adenine nucleotides adsorbed to charcoal according to the method of Crane and Lipmann (12). 1 ml. of a 25 per cent washed suspension of Norit A[1] was added to 5 ml. TCA extract and mixed thoroughly. In order to eliminate further a small amount of charcoal that persisted at the surface, 0.3 ml. ethanol was layered above the suspension. The suspension was centrifuged and washed 3 times by resuspending in 10 ml. water followed by centrifugation. The supernate and first washing, containing inorganic phosphate and phosphorylated intermediates, were combined and counted. The Norit A residue, containing adenine nucleotides, was resuspended in exactly 10 ml. water and thoroughly mixed with a pipet before sampling. One-half ml. samples were placed on planchets and dried at 80°C. Each result was the average of counts made on 4 to 8 planchets. The average deviation rarely exceeded ±5 per cent.[2]

Separation of Adenine Nucleotides.—Human kidney cells were collected from Roux bottles, resuspended, and treated with 10 μc./ml. P_i^{32} for 2 minutes as described in the preceding section. 4 to 5 mg. P_i^{32}-treated cells were rapidly chilled, centrifuged, washed, and extracted with 10 ml. cold 10 per cent TCA. 20 μg. each of cold carrier ADP (Pabst Laboratories) and ATP

[1] 10 gm. Norit A was suspended in 40 ml. distilled water, centrifuged, and washed 3 times in the centrifuge with 40 ml. changes of distilled water to remove fine particles tending to float at the surface. The residue was resuspended in 40 ml. water and placed on the shaking machine for 1 to 2 hours until a homogeneous suspension was obtained. It was again centrifuged and resuspended in 40 ml. water to give the stock 25 per cent suspension.

[2] Despite some difficulty in obtaining uniform sampling, we have found it preferable to count the suspensions directly rather than attempting to elute the nucleotides with hot HCl as recommended by Crane and Lipmann (12). The latter method has tended to yield low and variable results in our hands.

(Sigma, disodium salt, assay 98 to 100 per cent) were added to this extract.[3] The adenine nucleotides were then precipitated from the extracts with barium acetate following the method of LePage (13) and dissolved in 0.1 N HCl. Barium was removed by addition of a slight excess of sodium sulfate and the solution was immediately neutralized with 0.1 N NH_4OH. The solution containing the nucleotides was then passed through a 200 to 400 mesh Dowex 1 anion exchange resin (Cl^--form) column, 1 cm. × 1 cm^{-2}, at flow rates up to 1 ml. per minute according to the method of Cohn and Carter (14). The concentration of each effluent component was estimated from the absorption at 260 mμ. The specific activity of each component was expressed as P^{32} counts per minute per μM nucleotide per gram cell protein.

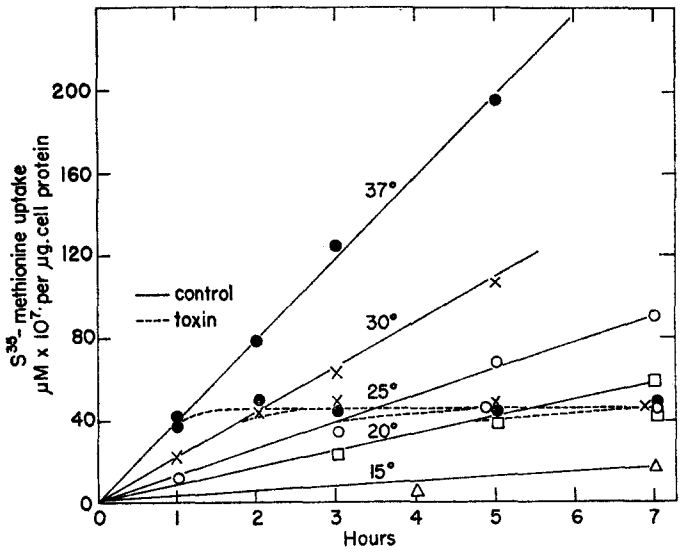

FIG. 1. Effect of diphtheria toxin on S^{35}-methionine incorporation into MK cells at various temperatures. Toxin was added to a final concentration of 1 Lf/ml. in each experimental flask.

RESULTS

Effect of Temperature on Protein Synthesis by Normal and Intoxicated Human Kidney Cells.—Fig. 1 shows the growth of MK cells at various temperatures between 15° and 37°C. as measured over a 7 hour period by the rate of incorporation of S^{35}-methionine into cell protein. At 37°C., this rate is equivalent to 40×10^{-7} μM methionine per μg. cell protein per hour; at 20°C. it is less than 8×10^{-7} μM/μg./hour. As shown by the dashed lines in Fig. 1, at any given temperature, incorporation of methionine continued at its normal rate in the presence of a saturating dose of diphtheria toxin until about $45 \pm 5 \times 10^{-7}$ μM had been taken up and then ceased altogether. Thus the latent period during which growth occurs at its normal rate increases from about 60 minutes at 37°C. to more than 18 hours at 15°C. in intoxicated cells.

[3] For the absolute determination of AMP, ADP, and ATP, extract from 11 mg. of cells was fractionated and no P_i^{32}, ADP, or ATP was added.

The growth rate at 37°C. for human kidney cells as measured by S^{35}-methionine uptake is about 80 per cent of that found by Strauss and Hendee (1) for HeLa cells and the action of a saturating dose of toxin on protein synthesis becomes apparent somewhat more rapidly than with HeLa cells. The latent period observed by Strauss and Hendee after adding toxin to HeLa cells was approximately 90 minutes at 37°C. as compared with 60 minutes for MK cells; 60 to 80 μM methionine were taken up before protein synthesis ceased in the former case as compared with 40 to 50 μM for MK cells.

The fact that, after addition of a saturating dose of toxin, incorporation of S^{35}-methionine into protein always continues until the same level is reached irrespective of temperature (*i.e.* 40–50 $\times$ 10^{-7} μM/μg. cell protein), and then ceases, suggests that possibly a precursor (in a very general sense) in protein synthesis must be used up before the toxin can exert its lethal effect. Such a precursor might be the amino acid pool, a stored energy source such as glycogen or fat, RNA, ATP, etc. Alternatively, the period of normal incorporation might represent the time required for accumulation of an intracellular inhibitor to reach a threshold toxic level.

That the precursor is not the amino acid pool is suggested by the following experiment. Washed MK cells were permitted to deplete their amino acid pool by shaking them at 37°C. suspended in Eagle's medium lacking all amino acids except S^{35}-methionine. After 2 hours methionine uptake fell to a low rate. Such cells immediately recovered their normal growth rate when resuspended in medium containing the full complement of amino acids. If the latent period of normal growth which follows addition of toxin represents the time required by the cells to exhaust their amino acid pool, we would expect a greatly shortened latent period after adding toxin to cells in which the pool is already depleted. This did *not* prove to be the case and MK cells depleted as above incorporated the usual 45 $\pm$ 5 $\times$ 10^{-7} μmoles methionine before protein synthesis ceased after they were resuspended in complete medium containing 1 Lf/ml. diphtheria toxin.

Does Toxin Enter the Cell during the Latent Period?—The molecular weight of diphtheria toxin is 70,000. It is difficult to conceive of any mechanism by which toxin molecules might enter intact growing cells, other than by some form of pinocytosis (15). Strauss and Hendee (1) found that the action of saturating levels of toxin on protein synthesis in HeLa cells could be completely reversed by antitoxin added during the first 30 minutes of the latent period and even when antitoxin was not added for 60 minutes following a saturating dose of toxin, the rate of S^{35}-methionine incorporation was only inhibited by 50 per cent, suggesting that one-half the cells still remained viable. We have found that intoxication of human kidney cells can also be reversed completely by antitoxin added within 30 minutes. Moreover if the cells are chilled 30 minutes after addition of toxin, centrifuged, and washed free of toxin with 3 changes of cold Hanks' solution, they continue to incorporate S^{35}-methionine

at the normal rate, at least for several hours, when resuspended in toxin-free complete medium at 37°C. These observations suggest that during the first 30 minutes of the latent period, no appreciable amount of toxin enters the cell. If any toxic action takes place during this period, it must be exerted externally at the cell surface.

Uptake of Potassium-42 by Normal and Intoxicated MK Cells.—As is well known, mammalian cells are able to transport and concentrate potassium ions so that intracellular potassium is high compared with its external concentration. (16). Using K^{42}, we have followed the potassium uptake by MK cells over a 2 hour period. Cells were suspended in the usual manner at 37°C. to a density of 5.2×10^5 per ml. in Eagle's medium containing 2 per cent dialyzed calf

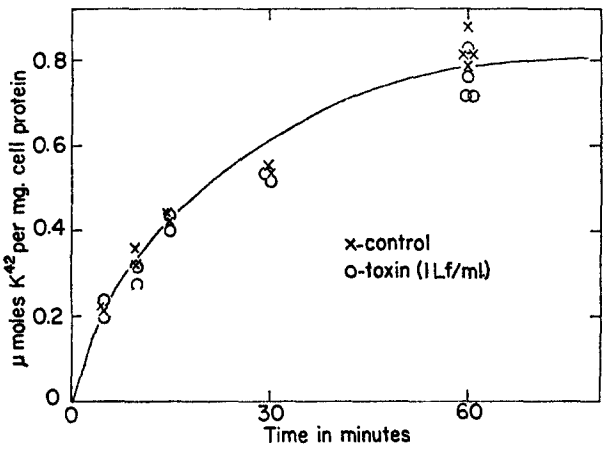

FIG. 2. Effect of diphtheria toxin on K^{42} uptake by MK cells at 37°C.

serum and 5.3 μmoles potassium per ml. At zero time a final concentration of about 1 μc./ml. $K^{42}Cl$ (so that the medium contained about 6×10^4 counts per μM K^+ per minute) was added to each of four flasks containing the suspended cells. Diphtheria toxin, 1 Lf/ml., was added to two of the flasks at zero time. 1 ml. samples were taken at intervals from each flask, rapidly chilled, centrifuged, and the cells washed twice with cold Hanks' solution (saline A). The sedimented cells were dissolved by treatment with two drops of normal KOH, made up to exactly 4 ml. with distilled water and 0.5 ml. aliquots were dried on planchets and counted. Fig. 2 shows that a saturating dose of toxin had no measurable effect on potassium uptake by MK cells for at least 1 hour. A steady-state, equivalent to about 0.8 μmoles K per mg. cell protein was reached within 60 to 90 minutes. Assuming that the cell volume is the same as for HeLa cells (120 mg. cell protein ≈ 1 ml. packed cells, Wu (17)), this steady-state level represents about a 20-fold concentration over the extracellular potassium level. Fig. 2 demonstrates, (unequivocally), that there was no leakage of K^+, at least

over a 1 hour period, and it would therefore appear most unlikely that toxin causes damage to the cell membrane during the latent period. Even after 2 hours, the intoxicated cells contained 80 per cent as much potassium as the control cells suggesting that injury to the membrane was only beginning at that time.

Effect of Toxin on Nucleic Acid Synthesis.—Recent studies by many workers have revealed that an intimate relationship exists between RNA and protein synthesis (18). It was therefore of interest to investigate the effect of diphtheria

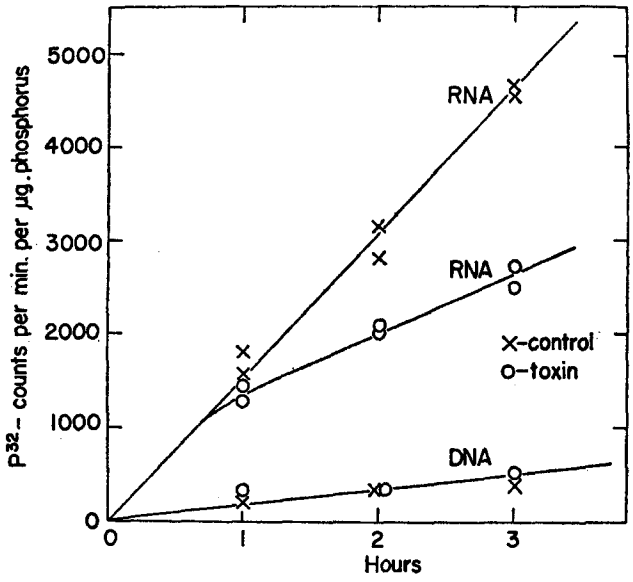

FIG. 3. Effect of toxin (1 Lf/ml.) on P_i^{32} incorporation into RNA and DNA by growing MK cells at 37°C.

toxin on the kinetics of nucleic acid synthesis by growing MK cells. Saturating doses of toxin were added to MK cells growing in suspension at 37°C. in Eagle's medium containing inorganic radiophosphate, P_i^{32}. At intervals, aliquots were taken, centrifuged, washed, and extracted with cold trichloroacetic acid. The extracts were fractionated for RNA-P^{32} and DNA-P^{32} as described in the section on methods. Fig. 3 shows that just as in the case of protein synthesis, ribonucleic acid synthesis continues at its normal rate for about 60 minutes after addition of a saturating dose of toxin. At this time P^{32} incorporation into RNA abruptly slows down to about 40 per cent of its initial rate. Fig. 3 shows that incorporation of P^{32} into DNA was not significantly affected by the toxin during the 3 hour experimental period.

The inhibition of RNA synthesis by toxin was confirmed by studying the

incorporation of 6-C^{14}–orotic acid as shown in Fig. 4. This pyrimidine is a precursor in the biosynthesis of uracil and is therefore taken up mainly by RNA but not DNA. Here again, it was found that radioactive orotic acid is taken up at the same rate by both control and intoxicated cells during the 1st hour. At this time orotic acid uptake by intoxicated cells abruptly slows down to less than 50 per cent of the initial rate.

Figs. 3 and 4 show that, in contrast to the incorporation of S^{35}-methionine into protein, the formation of labeled RNA does not cease altogether 1 hour

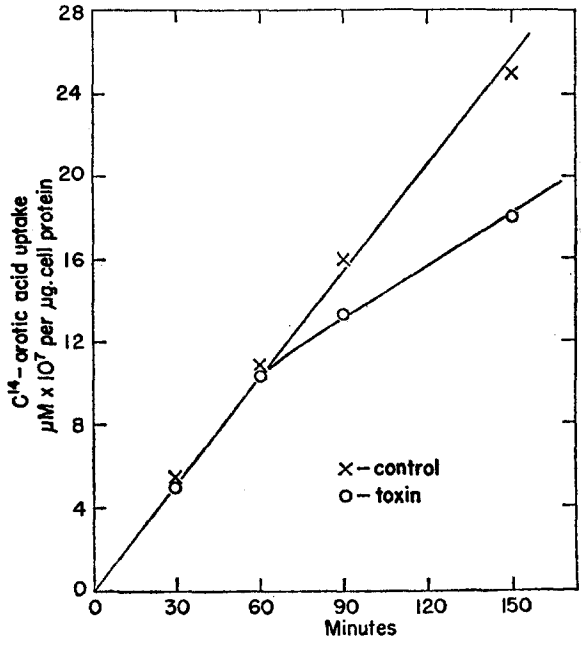

FIG. 4. Effect of toxin (1 Lf/ml.) on 6-C^{14}-orotic acid uptake by MK cells at 37°C.

after the addition of a saturating dose of toxin. At this time the rate changes and continues at 40 to 50 per cent of the initial rate, whether measured by incorporation of P_i^{32} into RNA or by 6-C^{14}–orotic acid uptake. Whether the continued incorporation of label into RNA, after protein synthesis has stopped, represents net synthesis or merely some kind of RNA turnover or exchange has not been determined.

Effect of Toxin on Adenine Nucleotide Formation.—When P_i^{32} is added to growing cells, it appears first in the adenine nucleotide fraction (mainly ATP) and later is transferred to hexoses, various metabolic intermediates, other nucleotides, RNA, lipid, etc. P_i^{32} was added to MK cells suspended in Eagle's medium at 37°C. and samples were taken at intervals over a 10 minute period, rapidly chilled, centrifuged, washed with Hank's solution, extracted with cold

TCA, and the radioactive charcoal-adsorbed adenine nucleotide fraction (12) was counted. In a second experiment the kinetics of incorporation of P_i^{32} into the same fraction was followed in cultures of cells treated 30 minutes previously with a saturating dose of diphtheria toxin. Fig. 5 shows that a steady state level of P^{32} incorporation into the ATP fraction was reached within 2 to 4 minutes in both cases, but this level was about 40 per cent lower in the presence of toxin. In all other experiments, a 2 minute treatment or "pulse" (19) was regarded as sufficient to reach a steady-state level of P_i^{32} incorporation into ATP. The same 40 per cent inhibition occurred when toxin was added only 5

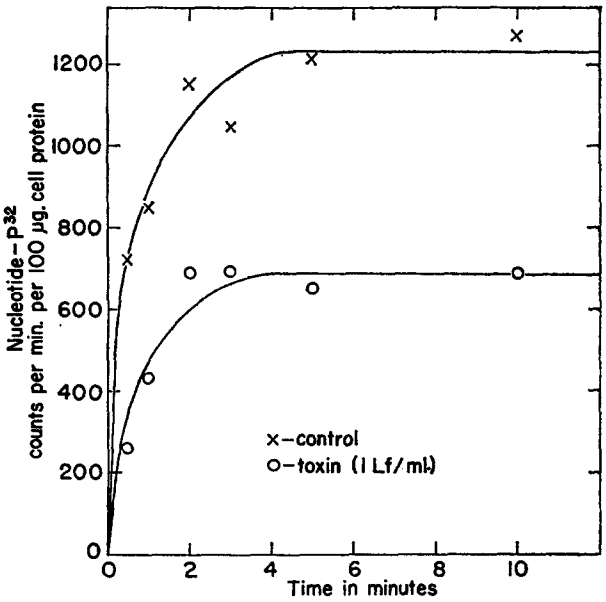

FIG. 5. Kinetics of P_i^{32} incorporation into adenine nucleotide by normal and intoxicated MK cells at 37°C. in Eagle's medium containing glucose.

minutes before the 2 minute P_i^{32} pulse. It will be recalled that all of the available evidence suggests that toxin does not enter the cell, at least not until near the end of the latent period. It would thus appear that the 40 per cent decrease in the steady-state level of incorporation of P_i^{32} into ATP is a *primary* effect of diphtheria toxin and is brought about by the toxin acting *externally* at the cell surface. It seems reasonable to conclude that the toxin interferes with transport of phosphate across the cell membrane. That the effect is specific is evidenced by the fact that 5 units antitoxin per ml. completely prevent the immediate inhibition by toxin of P_i^{32} incorporation into ATP.

Cellular Distribution of P^{32} after 2 Minute P_i^{32} Pulse.—About 8 per cent of the cold TCA-extractable phosphorus from MK cells treated 2 minutes with P_i^{32} is found in the adenine nucleotide fraction adsorbed to charcoal (see Table III).

The P^{32} remaining in the supernate has not been analyzed but presumably contains inorganic phosphate, hexose phosphates, and other phosphorylated intermediates. As can readily be seen from Fig. 3, incorporation into nucleic acid is negligible at this time.

The nucleotides from 11 mg. of MK cell protein were extracted with cold TCA, precipitated as barium salts, and then fractionated on Dowex-1 columns as described in the section on methods. The average yields recovered from 4 separate determinations were 11.0, 25.0, and 12.8 μmoles ATP, ADP, and AMP respectively per gm. cell protein. The cellular contents of the individual nucleotide components were determined at 30 and 90 minutes following ad-

TABLE I

Effect of Diphtheria Toxin on Adenine Nucleotide Content of Human Kidney (MK) Cells*

Nucleotide	Time				
	0 min.	30 min.		90 min.	
		Control	Toxin	Control	Toxin
AMP	14.5 13.8	13.6	12.3	10.6	12.3
ADP	27.0 24.0	25.0	22.6	24.0	27.0
ATP	10.2 11.1	10.4	8.8	11.7	6.6

* Each set of determinations on approximately 11 mg. cell protein. Toxin (1 Lf/ml.) added at zero time to experimental cultures. Results expressed as μM nucleotide per gram cell protein.

dition of a saturating dose of toxin. Table I shows that, within 90 minutes, toxin had caused a 40 per cent decrease in ATP content. There was no significant change in either ADP or AMP content during the 90 minute period.

After 2 minute treatment of cells with P_i^{32}, at least 80 to 85 per cent of all of the P^{32} found in the adenine nucleotide fraction is present in ATP. HeLa (5 mg. cell protein) and MK cells (4 mg. cell protein) were exposed to P_i^{32} for 2 minutes and then extracted with 10 per cent cold TCA. 20 μg. each of cold carrier ADP and ATP were added to the extract, the nucleotides were precipitated as barium salts and fractionated in the usual manner on Dowex 1 columns. The total radioactivity was then determined in each separated nucleotide fraction.[4] The ratio of P^{32} found in the ATP fraction to that found in the ADP

[4] The specific activity of the AMP fraction could not be determined because of contamination with P_i^{32}.

fraction was 5.7:1 and 4.6:1 for HeLa and MK cells respectively. Since MK cells contain 2.5 times as much ADP as ATP, the specific activity of P^{32} in ATP from MK cells is more than 12 times that in ADP after 2 minute treatment with P_i^{32}.

Effect of Toxin Concentration on ATP Formation.—The effect of varying toxin concentrations on inhibition of P_i^{32} incorporation into ATP is shown in Fig. 6. As little as 0.01 Lf/ml. (0.025 μg./ml.) cause a significant inhibition. It is of considerable interest that the minimum concentration of toxin required for maximal inhibition is 0.1 Lf/ml., a concentration of the same magnitude

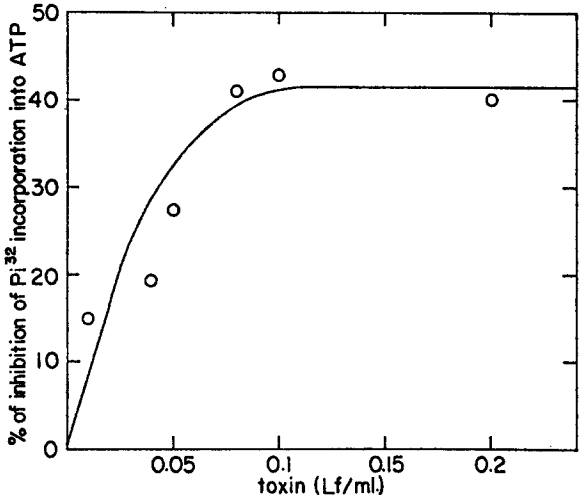

FIG. 6. Titration of inhibiting effect of diphtheria toxin on P_i^{32} incorporation into adenine nucleotide fraction of MK cells at 37°C.

as the minimal saturating dose of toxin determined by measuring S^{35}-methionine uptake. This is strikingly shown by comparing Fig. 6 with Table II.

Effect of Toxin on P_i^{32} Incorporation into ATP at Different Temperatures.—MK cells were treated with P_i^{32} for 2 minutes in the presence and absence of 1 Lf/ml. toxin at various temperatures. Cells were extracted with cold TCA in the usual manner and the P^{32} in the charcoal-adsorbed adenine nucleotide fraction and in the charcoal supernate was counted. The results are summarized in Table III. It will be seen that there is no effect of toxin on P_i^{32} uptake at 10°C. or below. At temperatures of 20°C. or higher, P_i^{32} incorporation into ATP is inhibited about 40 per cent. The difference between the P^{32} count found in the supernatant fraction from intoxicated cells as compared with the same fraction from normal cells, increases with increasing temperatures above 20°C. From Table I it can be calculated that the Q_{10} for P_i^{32} incorporation into cellular ATP is about 2.2 and that the activation energy or Arrhenius' constant E

has a value close to 14,000 calories per mole for both normal and intoxicated cells.

TABLE II

*Determination of "Saturating Dose" of Diphtheria Toxin for MK Cells**

Toxin	S^{35}-methionine incorporated per μg. cell protein	
	at 150 minutes	at 300 minutes
Lf/ml.	$\mu M \times 10^{-7}$	$\mu M \times 10^{-7}$
0	105	177
0.016	89	122
0.032	76	94
0.063	71	83
0.125	71	72
0.25	65	70
0.5	61	68
1.0	62	72

* S^{35}-methionine, to give a concentration of 10 μg./ml. and increasing concentrations of toxin were added at *zero* time to MK cells (202 μg. protein/ml.) in Eagle's medium lacking methionine. Cells from aliquots were collected and counted after 2.5 and 5 hours' shaking at 37°C.

TABLE III

*Effect of Diphtheria Toxin on Incorporation of P_i^{32} into Adenine Nucleotides at Different Temperatures**

Temperature	Control		Toxin (2.5 μg./ml.)	
	Nucleotide fraction (ATP)	Supernate	Nucleotide fraction (ATP)	Supernate
°C.				
3	1260	22,500	1200	22,500
10	1840	40,800	1950	39,000
20	4780	66,000	2720	59,500
30	7840	100,000	4940	80,000
37	17,100	200,000	10,680	154,000

* MK cells exposed 2 minutes to P_i^{32}. Activity is expressed as counts per milligram cell protein.

Effect of Toxin on Adenine Nucleotide Turnover.—Human kidney cells suspended in Eagle's medium at 37°C. were treated with P_i^{32} for 2 minutes, chilled, centrifuged, washed, and resuspended in the same medium but containing cold inorganic phosphate. After 30 minutes' shaking in the water bath at 37°C., 1 Lf/ml. toxin was added to one flask; a second flask served as a control. At intervals over a 5 hour period, samples were taken, the cells

collected, washed, extracted with cold TCA, and the P^{32} content of the charcoal-adsorbed adenine nucleotides was counted. The results of one of two such experiments is plotted in Fig. 7 and shows that P^{32} activity disappeared from the nucleotide fraction according to a first order process in both normal and intoxicated cells. However, the half-life of adenine nucleotide-P^{32} was more than 6 hours for intoxicated cells as compared with only 140 minutes for the control. Both curves shown in Fig. 7 extrapolate back to the same specific nucleotide-P^{32} activity at the moment of adding toxin, suggesting that the effect of toxin on nucleotide turnover is almost instantaneous.

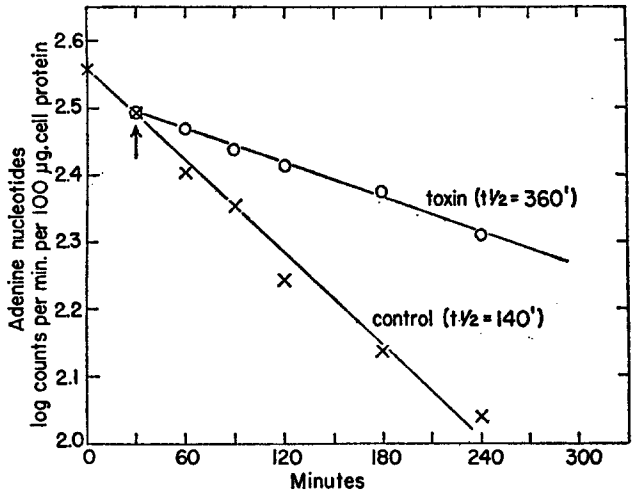

FIG. 7. Effect of toxin on adenine nucleotide turnover in MK cells. Washed cells, previously exposed for 2 minutes to P_i^{32}, were resuspended at 37°C. in Eagle's medium containing glucose. After 30 minutes, as indicated by the arrow, toxin was added to a concentration of 1 Lf/ml.

Effect of Certain Inhibitors on P_i^{32} Incorporation into ATP.—Table II shows that at a concentration of 2×10^{-4} µmoles/ml. antimycin A, an inhibitor which blocks electron transport through the cytochrome system by combining stoichiometrically with a factor lying between cytochromes *b* and *c* (20, 21), reduces the steady-state level of P_i^{32} incorporation into ATP in MK cells by 40 per cent, *i.e.* to the same extent as a saturating dose of toxin. Even at a concentration of only 2×10^{-6} µmoles/ml., antimycin A reduces the steady-state level by 20 per cent. The uncoupling agent dinitrophenol (DNP) at a concentration of 3×10^{-3} M also inhibits P_i^{32} incorporation to the same extent as toxin. However, a saturating dose of toxin, when added together with DNP, causes no further increase in the degree of inhibition. Table IV shows that inhibitors of the glycolytic process, such as iodoacetate (3×10^{-2} M) and fluoride (1×10^{-2} M) result in 56 and 90 per cent inhibition of P_i^{32} incorpora-

TABLE IV

Effect of Inhibitors on Steady State Level of P_i^{32} Incorporation into ATP

Inhibitor	Concentration	Inhibition of P_i^{32} incorporation into ATP	
		MK cells	Mouse spleen cells
		per cent	*per cent*
None		0	0
Toxin	0.1 Lf (0.25 μg.)/ml.	40	0*
Toxin (1 Lf/ml. + antitoxin (5 unit/ml.))	2.5 μg./ml.	0	—
Antimycin A	1.8×10^{-7} M	41	30
Antimycin A	1.8×10^{-9} M	20	0
Dinitrophenol (DNP)	3×10^{-3} M	37	30
Toxin (1 Lf/ml.) + DNP	3×10^{-3} M	40	—
Na iodoacetate	3×10^{-2} M	56	—
NaF	1×10^{-2} M	90	—
No glucose	—	38	30
No glucose, toxin	2.5 μg./ml.	40	—
No glucose, DNP	3×10^{-3} M	42	—

* No effect using 5 Lf (12.5 μg.)/ml. toxin.

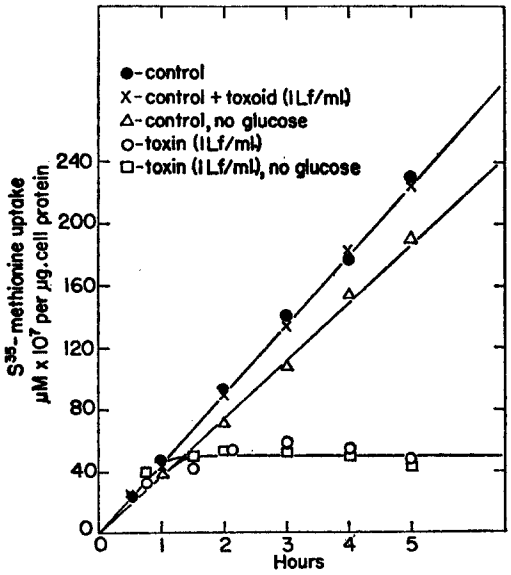

FIG. 8. Incorporation of S^{35}-methionine into protein by normal and intoxicated MK cells at 37°C. in the presence and absence of glucose. Note that purified toxoid (1 Lf/ml.) had no effect on methionine uptake.

tion respectively, suggesting that phosphate transport also depends in large measure upon glycolysis.

Table IV shows that the mere removal of glucose from Eagle's medium causes a 40 per cent decrease in the steady-state level of P_i^{32} incorporation into ATP. In the absence of glucose, neither toxin nor DNP influence the amount of P_i^{32} taken up during the 2 minute pulse to any measurable extent. Although toxin shows no immediate effect on ATP formation in the absence of glucose, Fig. 8 shows that its inhibitory effect on protein synthesis by MK cells does not depend upon the presence of glucose.

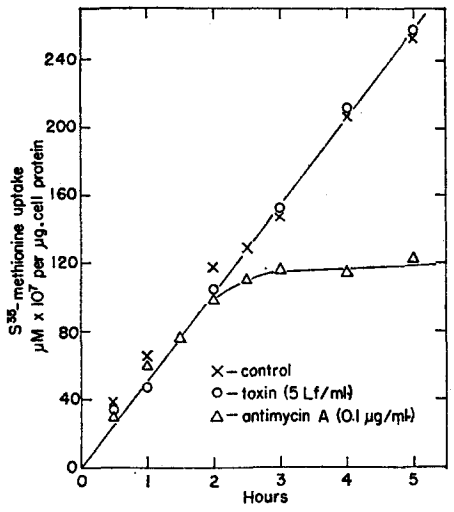

FIG. 9A

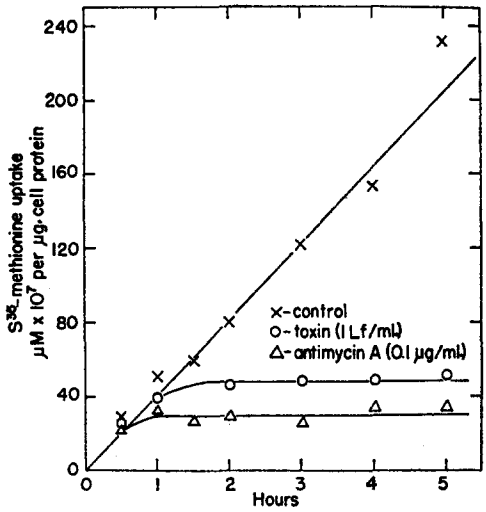

FIG. 9B

FIGS. 9A and 9B. Effect of diphtheria toxin and of antimycin A on S^{35}-methionine incorporation at 37°C. Fig. 9A, mouse spleen cells; Fig. 9B, human kidney (MK) cells.

Effect of Toxin on Mouse Spleen Cells.—It has been known for a long time that the mouse and rat are some 1000 times more resistant (on a weight basis) to the lethal action of diphtheria toxin than is the guinea pig, the human, or the rabbit. The last column of Table IV shows that even 50 times the saturating dose of toxin for human MK cells has no effect on P_i incorporation into mouse spleen cells tested under the same conditions. Moreover, this same high concentration of toxin (5 Lf/ml.) has no effect on S^{35}-methionine incorporation into mouse spleen cell protein, at least over a 5 hour period as seen from Fig. 9A. Thus the sensitivity of the whole animal to the toxin is reflected in the sensitivity of its cells growing in tissue culture.

Of the various inhibitors that we have studied, antimycin A most closely resembles diphtheria toxin in its action. It is of some interest that mouse spleen cells are considerably more resistant to inhibition by antimycin A than

are human kidney cells. Table IV shows that this antibiotic has no effect on P_i^{32} uptake by mouse cells at a concentration (1.8×10^{-9} M) that causes 20 per cent inhibition of MK cells. From Fig. 9A it can be seen that at the relatively high antimycin A concentration of 1.8×10^{-7} M, S^{35}-methionine uptake continued at the control rate for 2 hours and only ceased after 110×10^{-7} μmoles/μg. mouse cell protein had been incorporated. On the other hand, methionine uptake continued for only about 30 to 40 minutes and ceased when only 30×10^{-7} μmoles/μg. of human kidney cell protein had been incorporated.

DISCUSSION

In its action on susceptible animals, diphtheria toxin is characterized not only by its extreme toxicity, calculated to be only a few molecules per cell (22, 23), but also by a more or less prolonged latent period of many hours following its injection during which animals show no distress or other evidence of intoxication. Even after receiving several thousand lethal doses, a period of 5 to 6 hours must elapse before guinea pigs show the first signs of illness. If sacrificed during the latent period, neither biochemical nor morphological damage to tissue is evident. When cells from susceptible animals are grown *in vitro* in the presence of relatively high (saturating) concentrations of toxin, there is still a prolonged period before the cells show evidence of morphological change (Lennox and Kaplan (24); Placido Sousa and Evans (25)). Strauss and Hendee (1), working with HeLa cells and saturating doses of toxin, have reported that a few cells begin to show an abnormal appearance within 4 to 5 hours after which the numbers of visibly damaged cells increases rapidly. Strauss and Hendee have demonstrated that several hours *before* any generalized morphological damage becomes evident in toxin-treated cells, protein synthesis slows down abruptly and then ceases altogether. There still remains, however, a period of 60 to 90 minutes at 37°C. during which growth and S^{35}-methionine incorporation into cell protein continue at their normal rate. Strauss and Hendee showed further that during the first 60 minutes after adding a saturating dose of toxin, its action could be reversed by the addition of antitoxin and, from their studies on the kinetics of reversal, they postulated that there is a rapid adsorption of toxin to the cells, a process that is distinct from that causing later irreversible damage. The period during which S^{35}-methionine continues to be incorporated at its normal rate is longer at low toxin concentrations and more new protein is formed before incorporation ceases. As shown by the present studies, the duration of the latent period is also increased at lower temperatures, but the amount of amino acid incorporated into new protein in the presence of a saturating dose of toxin is independent of temperature.

Our studies have demonstrated for the first time what we believe to be a primary effect of the toxin. Inorganic phosphate is rapidly taken up by growing

mammalian cells and, within 2 to 4 minutes following addition of P_i^{32}, the isotope reaches a steady-state level in the adenosinetriphosphate fraction. We have found that if, within 5 minutes or less of adding a saturating dose of toxin, HeLa or normal human kidney cells suspended in medium containing glucose are treated with P_i^{32} for 2 minutes, there is a 40 per cent decrease in the level of P_i^{32} incorporation into ATP. This rapid inhibition of ATP formation by toxin can be prevented by specific antitoxin. The inhibition occurs at a time when intoxication is readily reversible and when the toxin can be removed by adding antitoxin or by mere washing of the cells. It may be of significance that the minimum concentration of toxin (0.1 Lf/ml.) required to bring about the maximum inhibition of ATP formation is very nearly the same as the minimal saturating dose of toxin measured by S^{35}-methionine uptake. Moreover, even at a concentration of 50 times its minimum saturating dose for MK cells, toxin has no effect on P_i^{32} incorporation into ATP by spleen cells from the mouse, an animal that is highly resistant to the lethal action of diphtheria toxin.

The relationship between the immediate reversible effect of toxin on ATP formation and its subsequent lethal action remains unexplained. In the absence of glucose the steady-state level of P_i^{32} incorporation into ATP is reduced by 40 per cent in normal cells and no effect of toxin on the steady-state level can be detected in glucose-free medium. Wu (17) has recently shown that HeLa cells frequently contain considerable quantities of glycogen. When placed in a medium containing no glucose, HeLa cells may continue to produce lactic acid aerobically for about 80 minutes at 37°C. before their glycogen stores become used up. However, we have found that MK cells in glucose-free medium continue to incorporate S^{35}-methionine into protein over a 5 hour period at an undiminished rate equal to about 85 per cent of the rate observed in the presence of glucose. Similar findings were recently reported by Quastel and Bickis (26) who showed that the efficiency with which a variety of tumor cells incorporate labeled glycine into their proteins as a result of the availability of ATP is approximately the same whether or not glucose is present. Although the inhibition by toxin of ATP formation by MK cells is only observed when glucose is present, its inhibitory effect on the incorporation of methionine into MK cell protein is not significantly different whether or not glucose is present.

Of the various inhibitors studied, antimycin A most closely resembles diphtheria toxin in its action. At a concentration of only 2×10^{-7} M, antimycin A causes an immediate 40 per cent inhibition of P_i incorporation into ATP by MK cells in glucose-containing medium. Just as in the case of toxin, addition of antimycin A to growing MK cells is followed by a period during which S^{35}-methionine continues to be incorporated into cell protein at its usual rate before protein synthesis ceases altogether and before its action becomes completely irreversible. In this respect, the action of the uncoupling agent,

dinitrophenol, differs from that of both toxin and antimycin A. Although DNP inhibits ATP formation to the same extent as toxin and antimycin, it does not completely arrest protein "turnover." In the presence of DNP, S^{35}-methionine uptake by MK cells continues at a rate equivalent to about 1 per cent of the total cell protein per hour even though net synthesis of protein appears to be completely inhibited (Eagle *et al.* (27); Strauss and Hendee (1)). Moreover, the action of DNP remains reversible for several hours and cells resuspended in medium free of DNP resume their normal rate of methionine uptake. Finally, the action of antimycin A resembles that of diphtheria toxin in its species specificity. In the presence of glucose, mouse spleen cells are far more resistant than are human kidney cells both to the immediate inhibition of P_i^{32} incorporation into ATP and to the delayed irreversible inhibition of S^{35}-methionine incorporation into protein caused by each of these cell poisons. It would thus seem likely that both of these toxic substances exert a primary effect at the cell surface before penetrating into the interior of the cell.

Antimycin A is a powerful non-competitive inhibitor of electron transport *via* the cytochrome system and acts by combining with a component lying between cytochromes *b* and *c*. While it seems virtually certain that toxin has no direct effect on electron transport, it is quite possible that it may interfere with cytochrome-linked oxidative phosphorylation (Pinchot and Bloom (28); Pinchot (29); Pappenheimer, (30)). It will be recalled that experiments on the effect of diphtheria toxin on the *Cecropia* silkworm led to the suggestion that its most probable action in this species was to interfere in some manner with the normal function of cytochrome *b* or of a factor between *b* and c_1 (Slater factor) (30).

In conclusion it is tentatively suggested that diphtheria toxin exerts its primary effect at the cell surface by inhibition of cytochrome-linked oxidative phosphorylation concerned with transport of inorganic phosphate across the cell membrane. What relation this primary *reversible* effect bears to the sequence of events leading to eventual death of the cell 1 or more hours later remains to be determined by future experiments.

SUMMARY

At concentrations of 0.1 Lf (0.25 μg.)/ml., or greater, diphtheria toxin produces an immediate decrease (40 ± 5 per cent) in the steady-state level of incorporation of inorganic phosphate into ATP by cultures of normal human kidney cells growing at 37°C. in the presence of glucose. The effect is readily reversible by specific antitoxin for a period of 30 minutes or more after adding toxin. Protein synthesis in these cells continues at a normal rate for 60 minutes after adding toxin and the toxin-treated cells are able to take up and concentrate potassium ions normally for at least 1 hour. Even high concentrations of toxin (5 Lf/ml. or more) fail to effect either protein synthesis or ATP formation

by cultures of spleen cells from the mouse, an animal that is relatively resistant to the lethal action of the toxin. Of various inhibitors studied, antimycin A most closely resembles toxin in its action, both on protein synthesis and on ATP formation. Mouse cells are considerably more resistant to antimycin A than are human kidney cells.

Human kidney cells treated with saturating doses of toxin continue to form RNA at a normal rate for about 1 hour, after which the rates of both P_i^{32} incorporation and of 6-C^{14}-orotic acid incorporation into RNA sharply decline and continue at about 40 to 50 per cent their initial rate. At 37°C., S^{35}-methionine incorporation into cell protein ceases altogether 60 to 75 minutes after addition of a saturating dose of toxin.

The effect of saturating doses of toxin on S^{35}-methionine incorporation into human kidney cell protein at different temperatures, has been studied.

It is concluded from the present studies that diphtheria toxin exerts a primary, *reversible* effect at the surface of susceptible cells where it inhibits cytochrome-linked phosphorylation concerned with transport of inorganic phosphate across the cell membrane.

We are indebted to Dr. William R. Sistrom for numerous helpful suggestions.

Addendum.—After the preparation of this manuscript had been completed, we became aware of the studies of Strauss (*J. Exp. Med.*, 1960, **112,** 351) showing that incorporation of tritiated thymidine and uridine into HeLa cell nucleic acid is less sensitive to inhibition by diphtheria toxin than is S^{35}-methionine incorporation into protein. The experiments of Strauss on P^{32} leakage have indicated that toxin causes no damage to the membrane of HeLa cells for at least 1 to 2 hours. This conclusion is in agreement with our own findings using K^{42} and MK cells.

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