STUDIES ON THE MODE OF ACTION OF DIPHTHERIA TOXIN*

I. Phosphorylated Intermediates in Normal and Intoxicated HeLa Cells

By R. J. COLLIER,‡, § Ph.D., and A. M. PAPPENHEIMER, Jr., Ph.D.

(From the Biological Laboratories, Harvard University, Cambridge, Massachusetts)

(Received for publication, July 20, 1964)

It was shown by Strauss and Hendee (1) that when purified diphtheria toxin was added to cultures of HeLa cells to a level of 0.5 μg per ml or greater, incorporation of S^{35}-methionine into cell protein continued at a normal rate for 1.5 to 2 hours and then ceased abruptly. In the presence of lower concentrations of toxin, protein synthesis continued at its normal rate for a longer period before coming to a standstill. These findings were confirmed by Kato and Pappenheimer (2) who observed a simultaneous reduction in the rate of ribonucleic acid formation. Kato and Pappenheimer also reported that within a few minutes after addition of a saturating dose of toxin to cultures of either human MK cells or HeLa cells growing in the presence of glucose, there was a 40 per cent inhibition in the "steady state" level of incorporation of inorganic phosphate into adenine nucleotides. They suggested that diphtheria toxin acts reversibly at the cell surface where it interferes in some way with cytochrome-linked phosphorylation concerned with transport of inorganic phosphate across the cell membrane. It was postulated that this in turn leads to the arrest of protein synthesis. Strauss (3), working independently, also observed some decrease in nucleic acid synthesis following the arrest of protein synthesis in intoxicated cells. However, he was unable to demonstrate any significant difference between normal and intoxicated cells in the kinetics of incorporation of inorganic P^{32} into nucleotides over a period of at least 120 minutes following the addition of a saturating dose of toxin.

If inhibition of protein synthesis in toxin-treated mammalian cells is, in

* This investigation was aided by a grant from the National Science Foundation. The work was conducted in part under the sponsorship of the Commission on Immunization, Armed Forces Epidemiological Board and supported in part by the Office of the Surgeon General, Department of the Army.

‡ This work was carried out during tenure by Dr. Collier of a Graduate Fellowship of the National Science Foundation.

§ Present address: Institute of Molecular Biology, University of Geneva, Geneva, Switzerland.

fact, caused by interference with adenine nucleotide formation, we would expect to find a marked reduction in the ATP content of intoxicated cells. In the present paper we are reporting determinations of the ATP contents of normal and intoxicated HeLa cells growing in the presence and in the absence of glucose. The results have revealed that no significant change occurs in the cellular levels of ATP, GTP, or hexose phosphates for a period of at least 5 to 6 hours after addition of a saturating dose of diphtheria toxin. Thus the earlier report from this laboratory (2) has *not* been confirmed, and it now seems virtually certain that the arrest of protein synthesis in intoxicated mammalian cells is not due to inhibition of energy metabolism by the toxin.

Materials and Methods

Cell Line.—A subclone of strain S-3 HeLa cells, adapted for growth in spinner culture, was used in all experiments. The cells were cultivated as a routine at 35–37°C in suspension and were maintained in the range of 1×10^5 to 4×10^5 cells per ml. The cultures remained in the logarithmic growth phase at least between the concentrations of 1.8×10^5 and 3.8×10^5 cells per ml.

Growth Medium.—Cells were cultivated on Eagle's medium (4) containing 5 per cent horse serum. The spinner culture apparatus and methods of culture used were similar to those described by McLimans *et al.* (5).

Diphtheria Toxin.—The diphtheria toxin used in these studies was prepared according to the method of Yoneda (6) as modified by Miss P. A. Miller[1] of this laboratory. Initial purification of the toxin was carried out by ammonium sulfate fractionation, the fraction between 45 and 60 per cent saturation being retained. This fraction was redissolved in 0.01 M phosphate buffer, pH 6.9, and the ammonium sulfate was removed by passing the solution through a G-50 sephadex column equilibrated with the same buffer. Subsequently the toxin was applied to a column of DEAE cellulose and eluted with increasing concentrations of potassium phosphate, pH 6.9. The toxin peak, which was eluted at 0.1 M phosphate, was concentrated by dialysis against a 67 per cent saturated solution of ammonium sulfate, and the resulting precipitate was redissolved and dialyzed against 0.01 M tris, pH 7.3. The final product contained approximately 2.6 μg protein per Lf unit and about 60 M.L.D. per Lf.

When the toxin was diluted to 10 Lf/ml or less, a diluent of 0.01 M tris buffer containing 100 μg bovine serum albumin per ml was used, in order to eliminate the possibility of surface denaturation.

Diphtheria Antitoxin.—Antitoxin 1520-5353AD was supplied by Lederle Laboratories, Pearl River, New York. This antitoxin is a pepsin-digested horse pseudoglobulin fraction which shows only one minor band (in addition to the strong toxin band) upon immunoelectrophoresis against crude toxin preparations (7). It was exhaustively dialyzed before use against 0.01 M tris buffer, pH 7.3, and was stored frozen.

Reagents and Enzymes.—Reduced nicotinamide adenine dinucleotide (NADH) and hexokinase were purchased from Sigma Chemical Company, St. Louis. UTP, GDP, and GTP were products of Calbiochem Company, Los Angeles. ATP was supplied by Pabst Laboratories, Milwaukee, Wisconsin. Glucose-6-phosphate was obtained as the barium salt from Mann Research Labs., New York. It was converted to the sodium salt by treatment with sodium sulfate prior to use.

[1] We are grateful to Miss Pauline A. Miller who prepared and purified the toxin used in these studies.

The enzyme fractions used in assaying ATP and hexose phosphates were prepared from rabbit muscle according to the method of Racker (8). A rabbit was bled to death by heart puncture and the skeletal muscle was removed. The muscle was passed through a meat grinder and extracted with 0.003 N KOH, and the crude extract thus obtained was fractionated by ammonium sulfate precipitation. The fraction precipitated between 50 and 70 per cent saturation with ammonium sulfate, contained the enzymes aldolase, triosephosphate isomerase, and α-glycerolphosphate dehydrogenase. This "aldolase" fraction was not purified further, and was stored frozen as a thick paste. The fraction precipitated between 20 and 50 per cent saturation with ammonium sulfate containing most of the phosphohexokinase (PHK) activity. This fraction was further purified by ammonium sulfate precipitation; it was found that the 36 to 49 per cent fraction contained the highest specific activity of phosphohexokinase. This "PHK" fraction was stored frozen in paste form.

Radioisotopes.—Carrier-free, radioactive orthophosphate (P_i^{32}) was obtained from Iso-Serve, Inc., Cambridge, Massachusetts. Before use, it was heated to 100°C in 1 N HCl and maintained at that temperature for 10 minutes in order to hydrolyze any pyrophosphate present. S^{35}-methionine was purchased from Abbot Laboratories, North Chicago, and C^{14}-phenylalanine from Schwarz BioResearch, Orangeburg, New York.

Protein and Cell Density Determinations.—Protein determinations were carried out on washed 1 ml samples of cells by the Oyama and Eagle modification (9) of the Lowry method (10). Cell density was more conveniently estimated by measuring the optical density of cultures at 700 mμ (OD 700). At this wavelength the growth medium has an absorbance of zero, and it was found that the OD 700 due to scattering of light by the cells gave a direct measure of cell protein, at least within the range from 1×10^5 to 4×10^5 cells per ml. Using 1 ml cuvettes in a Zeiss spectrophotometer model PMQ II, an OD 700 of 0.4 was found to correspond to approximately 155 μg cell protein per ml. Multiplication of the OD 700 by the factor 1.1×10^6 gave a good estimate of the number of cells per ml.

Lactic Acid.—Lactic acid was determined by the method of Barker and Summerson (11).

Protein Synthesis in Normal and Intoxicated Cells.—In order to be certain that toxin did actually block protein synthesis, 15-ml samples were removed at the beginning of each experiment and the uptake of a labeled amino acid by the cells was followed. From each culture to be analyzed for ATP, samples were taken directly after adding either toxin or toxin plus antitoxin. They were incubated in a shaking water bath at 37° C in 50-ml flasks containing either S^{35}-methionine or C^{14}-phenylalanine. The amino acid uptake was followed as described by Strauss and Hendee (1). In no case was there any further incorporation of label by the intoxicated cells after 3 hours, although uptake continued at the normal rate in the control flasks.

In one experiment involving ion exchange analysis of ATP and GTP, in which the cells were heavily labeled with P^{32}, each sample was placed in a liquid scintillation vial and stored at −20° C for approximately 4 months to allow the P^{32} to decay to a low level of activity. At the end of this period each vial was filled with scintillation fluid having a dioxane base, and counted for C^{14} in a Packard tri-carb liquid scintillation spectrometer. The radioactivity from P^{32} was found to be negligible at this time.

Preparation of Cell Extracts.—When the enzymatic method of analysis of ATP and hexose phosphates was employed (12) samples of approximately 40 ml each were removed at intervals from control and toxin-containing cultures. Each sample was chilled immediately to 0–5° C, and after determination of the exact volume, was centrifuged at 1500 RPM for 5 minutes at 0°C. The supernate was decanted, and the cell pellet was then extracted with 1.0 ml of 5 per cent perchloric acid. After centrifugation, the supernate was carefully removed, and the precipitate was reextracted with 0.3 ml perchloric acid. The combined supernates were then neutralized with KOH, using phenol red as an indicator, and after 30 minutes at 0° C, the

potassium perchlorate precipitate was removed and reextracted with water. Finally, the total volume of each extract was adjusted to 4.0 ml. The extracts were stored frozen until analyzed.

In experiments involving the chromatographic method of analysis of ATP and GTP (13, 14), cultures of cells heavily labeled with P_i^{32} were used. Samples of 10 ml were removed from each flask, and after cooling to 0°,C were centrifuged at 1500 RPM in the cold. Each cell pellet was then extracted with 1.0 ml of 8 per cent trichloroacetic acid (TCA), and exactly 0.5 ml of a solution of non-radioactive ATP and GTP, at concentrations of 16.6 and 3.5 mM respectively, was added immediately to each tube. After thorough mixing, the tubes were centrifuged and the supernates collected. The supernates were then extracted six times with ether to remove the TCA, bringing the pH to about 3.5, and were frozen until analyzed.

Enzymatic Analysis of ATP and Hexose Phosphates.—Analysis of ATP and hexose phosphates was carried out enzymatically by the method of Slater (12). This method involves measurement of hexose monophosphates (HMP) and hexose diphosphates (HDP), using glycolytic enzymes to convert these to dihydroxyacetone phosphate, and the enzyme α-glycerolphosphate dehydrogenase to reduce the dihydroxyacetone phosphate to α-glycerolphosphate with the concomitant oxidation of NADH. The oxidation of NADH to NAD is followed by measurement of optical density at 340 mμ. Since the equilibrium of the oxidation-reduction reaction is greatly in favor of the products, NAD and α-glycerolphosphate, the extent of NADH oxidation is a measure of dihydroxyacetone phosphate, and consequently also of HDP.[2] In the presence of excess added ATP, HMP is converted to HDP by phosphohexokinase, and NAHD oxidation is a measure of HDP + HMP. If, instead of ATP excess HMP is added, the extent of the reaction is a measure of HDP plus the total high energy phosphate bonds (~P) in ATP and ADP. ADP reacts according to the reaction, 2 ADP → ATP + AMP, because of myokinase present in the aldolase fraction. For determination of ATP, the ATP in an aliquot of the test sample is first allowed to react with glucose in the presence of hexokinase to form an equivalent amount of glucose-6-phosphate. After inactivation of the hexokinase by boiling, the glucose-6-phosphate is assayed as HMP in the above assay system. In this case the NADH oxidized is a measure of ATP + HMP + HDP. Thus, for each sample four separate measurements are made: P + HDP, ATP + HMP + HDP, HMP + HDP, and HDP. Values for ATP, HMP, ~P, and ADP[3] can be obtained by subtraction of the appropriate measured values. Both the aldolase and PHK enzyme fractions are used in all of the spectrophotometric assays except that for HDP, in which the PHK fraction is omitted.

The compositions of reaction mixtures and details of experimental procedure described by Slater (12) were followed exactly in these assays. Kinetic optical density measurements of NADH oxidation were recorded directly by means of a Leeds Northrup strip chart recorder with the input signal supplied by an absorbance indicator and optical density convertor manufactured by Gilford Instrument Company, Oberlin, Ohio. The Gilford absorbance indicator was connected in place of the phototube assembly to a Beckman DU monochromator. An automatic cuvette changer, also manufactured by Gilford, permitted the optical densities of three samples to be recorded in the same run.

Chromatographic Analysis of ATP and GTP.—In two experiments the intracellular levels of ATP and GTP were measured using ion exchange chromatography. Extracts were prepared from HeLa cells labeled with P^{32}, and after adding known amounts of non-radioactive carrier ATP and GTP, the latter nucleotides were isolated by use of dowex-1 anion exchange columns. From the specific activity of P^{32} in each isolated nucleotide, the cellular levels of ATP and GTP could be calculated.

---

[2] Hexose phosphates and triose phosphates cannot be distinguished by this assay. For convenience, the sum of the two is referred to as HDP.

[3] Because of the low ADP content in HeLa cell extracts, values for ADP = ~P − 2 ATP fluctuated widely and are of doubtful significance.

A method adapted from that of Hurlbert *et al.* (13) was used to separate ATP and GTP from the other components of the cell extracts. Elution with the volatile salt, ammonium bicarbonate, as described by Cohn and Bollum (14), was incorporated into the procedure. A 2 ml column of washed dowex-1 X8 200 to 400 mesh, in the chloride form, was converted to the carbonate form by washing with 1 M Na_2CO_3 until no chloride ions could be detected in the eluate. It was then washed with distilled water until the pH of the eluate reached neutrality. One mg of UTP was added to 1.5 ml of the cell extract to be analyzed, and this was then allowed to run slowly onto the column without pressure. The added UTP served merely as a marker in the elution procedure. The column was then washed with approximately 10 ml of distilled water, and elution was begin with 0.3 M ammonium bicarbonate. The supply of eluant was elevated above the column to provide hydrostatic pressure sufficient to produce a flow rate of 0.5 to 1.0 ml per minute. Samples of 150 drops each were collected on an automatic fraction collector, and the absorption of each fraction was checked at 280 and 260 mμ. When the peak of UTP (detected by its 280/260 absorption ratio of about 0.4) had been eluted, the only nucleotides remaining on the column were GDP, ATP, and GTP. These were then removed with approximately 20 ml of 1 M ammonium bicarbonate. The eluate containing GDP, ATP, and GTP was then lyophilized to remove the water and ammonium bicarbonate; the residue was dissolved in 2.5 ml distilled water in preparation for the separation of these three nucleotides on a second column.

A 2 ml column of dowex-1 in the chloride form was converted to the formate form with 1 M ammonium formate in a manner similar to that described above, and the column was then rinsed with CO_2-free (freshly boiled) distilled water. One mg of GDP was added as an elution marker to 1.5 ml of the GDP, ATP, GTP mixture from the previous column, and this was then allowed to run slowly onto the column. The column was washed with a few ml of CO_2-free water, and elution was begun with a solution of 4 N formic acid-0.1 M ammonium formate. As soon as the peak of GDP (280/260 ratio = 0.66) had passed off the column, the eluant was changed to 4 N formic acid-0.2 M ammonium formate. Elution was continued with this solution until all ATP had been removed from the column. Finally, GTP was eluted with a solution 4 N in formic acid and 0.4 M in ammonium formate.

Optical density measurements were made at 259 mμ for tubes containing ATP and at 252 mμ for those containing GTP. Blanks of the appropriate eluting solutions were subtracted in each case. Passage of solutions of formic acid and ammonium formate through blank columns did not cause significant increase or decrease in their absorption at these wavelengths.

Radioactivity measurements were carried out on duplicate or triplicate 0.5 ml samples from every tube containing ATP or GTP, and from tubes on both sides of the nucleotide peaks. The samples were plated on aluminum planchets, dried, and then counted in a thin window, gas flow automatic counter. After appropriate corrections for counter efficiency and time decay of the P^{32}, the specific activity of P^{32} in each of the nucleotides at the time of the experiment could be calculated.

RESULTS

Effect of Toxin on P_i^{32} Incorporation into Cell Nucleotides.—Before proceeding to the study of the effect of toxin on the cellular levels of individual nucleotides, numerous attempts were made to repeat the pulse-labeling experiments reported by Kato and Pappenheimer (2) on the kinetics of incorporation of P_i^{32} into charcoal-adsorbable nucleotides by normal and intoxicated mammalian cells. Despite the fact that the conditions duplicated those of the previous study as closely as possible, no steady state level of P_i incorporation was reached over a 20 minute period. Moreover, the P_i^{32} uptake into the charcoal-adsorbable

fraction remained linear and there was no significant difference between normal and toxin-treated cells. Thus our results fail to confirm those reported earlier from this laboratory and agree closely with those of Strauss (3), who observed almost identical, linear kinetics of P_i incorporation into nucleotides over a 40 minute period both in the presence and absence of toxin.

Enzymatic Assay of ATP and Hexose Phosphates in Normal and in Intoxicated Cells.—In a typical experiment, a spinner culture containing 2.4×10^5 cells per ml was divided into three portions. Into flask C (control) was placed

TABLE I

ATP and Hexose Phosphate Levels in Intoxicated and Non-Intoxicated HeLa Cells as Determined by Enzymatic Method of Assay*

	Time	I	II	III	IV	V	VI
		~P + HDP	ATP + HDP + HMP	HDP + HMP	HDP	~P‡	ATP§
	hrs.						
No toxin or antitoxin	0	44.9	30.1	11.3	6.6	38.3	18.8
	0	47.5	32.6	12.1	7.1	40.4	20.5
	0.5	51.5	32.1	10.0	7.5	44.0	22.0
	2.0	47.8	31.7	9.1	9.9	37.9	22.6
Toxin	0.75	50.8	30.6	10.5	9.7	41.1	20.1
	1.5	50.4	33.0	10.5	10.5	39.9	22.5
	4.5	50.3	32.6	12.8	9.8	40.5	19.8
Toxin + antitoxin	0.75	51.5	31.8	9.9	7.4	44.1	21.9
	1.5	49.0	33.0	9.5	9.0	40.0	23.5
	5.0	49.7	30.1	11.4	8.7	41.0	18.7

* All values expressed as μmoles per gram cell protein.

‡ Obtained by subtraction of column IV from column I.

§ Obtained by subtraction of column III from column II.

125 ml of culture and 185 ml was placed in each of the flasks T and AT. A spinner magnet was inserted into each flask and all three were placed at 37°C over magnetic stirrers. Toxin was added to flask T to a level of 2.3 Lf per ml, and to flask AT was added the same amount of toxin which had been preincubated with 10 units per ml of antitoxin for 1 hour at 37°C. Fifteen ml was removed immediately from each flask and S^{35}-methionine was added for determination of the kinetics of protein synthesis. Three samples of 40 ml each were removed at intervals over a 5 hour period, and the cells were extracted and analyzed for ATP and hexose phosphates as described under Materials and Methods.

The results of this experiment are shown in Table I. For none of the parame-

ters measured, including ATP, hexose monophosphates (HMP), hexose diphosphates (HDP), and total high energy phosphate (~P), does there appear to be a significant difference between the control and toxin-containing flasks, even long after synthesis of protein ceased in the flask with toxin. The kinetics of incorporation of S^{35}-methionine into protein by samples from the T and AT flasks were identical with those found by Strauss and Hendee (1) and Kato and Pappenheimer (2). Protein synthesis ceased in flask T, 2 to 2.5 hours after toxin was added.

Almost identical results were observed in five other experiments of this type. For the most part the only changes made in the various experiments were in the times of sampling and in the nature of the controls. A maximum of three samples was usually taken from each flask in any one experiment, and since the times of sampling were varied with each experiment, samples have been removed within each hour interval over a 5 to 6 hour period after addition of toxin. No significant decline in either ATP or hexose phosphates was detected at any time in these experiments.

Comparison of the values for ATP obtained here with those reported by others is of some interest. The ATP values reported here are nearly double those reported by Wu for HeLa cells (15), and Kato and Pappenheimer for MK cells (2). Our ADP values, on the other hand, are significantly lower than those obtained by these two groups. The exact reason for this difference is not known, but it may be related to the way in which the cells were treated in preparation for experiments. The previous workers used cells which had been grown in monolayer culture. To prepare suspensions of these cells, it was first necessary to remove them from glass by treatment with either trypsin or the chelating agent EDTA. The trypsin or EDTA was then removed by repeated centrifugation and resuspension of the cells in medium. Such procedures traumatize the cells to some extent and might produce significant alterations in the relative levels of ATP and ADP. Since our cells were grown in suspension, it was not necessary to subject them to such treatment. All the experiments reported here were carried out with exponentially growing cells; they were not centrifuged or resuspended in fresh medium before experiments were begun. The high ATP values shown by our results may, alternatively, be typical of the particular strain of cells we have used.

Enzymatic Analysis of ATP in the Absence of Glucose.—The above results cast serious doubt on the earlier suggestion (2) that the production of ATP is affected by diphtheria toxin. However, it was felt that the large glycolytic production of ATP by these cells (16) might possibly be masking an effect of the toxin on oxidative ATP production. In the absence of glucose it would be expected that this masking would be greatly reduced, and an effect of toxin on the oxidative production of ATP would be amplified.

Two equal portions of growing cells were centrifuged and washed twice

with glucose-free medium. One was resuspended in glucose-free medium and the other in medium containing the normal concentration of glucose. Each of these suspensions was once again divided in two, and the four flasks were placed at 37°C for 2 hours. Toxin was then added to a level of 2.7 Lf per ml to one flask with and to one flask without glucose. Samples were removed immediately from all four flasks, and S^{35}-methionine incorporation into cell protein was followed in each. Enzymatic analysis of ATP and hexose phosphates was carried out on two 40 ml samples removed from each flask at intervals over a period

TABLE II

ATP and Hexose Phosphate Levels in Intoxicated and Non-Intoxicated HeLa Cells in the Presence and Absence of Glucose*

	Toxin	Time	I	II	III	IV	V	VI
			~P + HDP	ATP + HDP + HMP	HDP + HMP	HDP	~P‡	ATP§
		hrs.						
Glucose	0	1.0	46.5	25.0	4.9	2.3	44.2	21.0
	0	5.0	51.5	25.0	7.0	4.5	47.0	18.0
	+	2.5	52.0	25.8	5.8	3.1	48.9	20.0
	+	5.5	60.4	32.9	8.6	3.2	57.2	24.4
No glucose	0	1.0	32.8	19.8	1.9	1.9	30.9	17.9
	0	5.0	38.1	15.5	1.5	0.9	37.2	13.9
	+	2.5	39.8	17.7	2.1	0.7	39.2	15.6
	+	5.5	42.0	18.0	0.7	0.9	41.0	17.3

* Determined by the enzymatic method of Slater (12). All values expressed as μmoles per gram cell protein.

‡ Obtained by subtraction of column IV from column I.

§ Obtained by subtraction of column III from column II.

of 5 hours after the addition of toxin. Lactic acid production was followed in samples taken hourly.

Table II shows the results of this experiment. The cellular level of ATP was found to be about 20 per cent lower in the absence of glucose. On the other hand, the levels of hexose phosphates showed a more marked drop, as would be expected. Toxin caused no additional decline in ATP or any of the other intermediates, either in the presence or absence of glucose. As expected, incorporation of S^{35}-methionine into protein ceased in both flasks containing toxin about 2 hours after its addition. Finally, less than 4 per cent as much lactic acid was produced in the absence of glucose as in its presence.

From these results it would seem highly unlikely that glycolytic production of ATP masks an effect of toxin on oxidative ATP production. Even when

glycolysis is cut to less than 4 per cent of normal, the cellular ATP level remains high in the presence of toxin for at least 2 hours after protein synthesis has ceased.

Analysis of ATP and GTP by Anion Exchange Chromatography.—The results of the enzymatic analyses of ATP in intoxicated and non-intoxicated cells were checked by using a totally unrelated method of analysis. Cells which had been heavily labeled with P^{32} were incubated with toxin, and samples removed periodically were extracted and analyzed for ATP content by means of anion exchange chromatography. GTP could also be isolated without difficulty in the same experiments and was determined because of its importance in protein synthesis (17).

Cells were grown for 40 hours on Eagle's medium containing P_i^{32} at a specific activity of 6.0 μc per μmole. The medium contained inorganic phosphate at a concentration of 1.1 mM, which is one-tenth the concentration used normally in spinner cultures. The phenylalanine concentration was one-fourth that normally used. It had been found previously that the generation time of these cultures was not altered significantly when they were grown in a medium containing the above concentrations of phosphate and phenylalanine. The culture was divided into two equal portions, and toxin was added to one of them to a level of 2 Lf per ml. Fifteen ml was then withdrawn from each flask, and C^{14}-phenylalanine was added for determination of the kinetics of protein synthesis. At intervals over a period of 5 hours, 3 samples were taken from each flask for determination of ATP and GTP. After chilling and centrifugation the cells were extracted with TCA. Known amounts of non-radioactive carrier ATP and GTP were then added, and the extracts were prepared for chromatography by extracting with ether to remove the TCA.

Isolation of ATP and GTP in these extracts was carried out by chromatography on dowex-1 ion exchange columns as described under Materials and Methods. Aliquots of column eluate from tubes containing the ATP and GTP fractions were analyzed for radioactivity, and nucleotide concentrations were determined by measurement of optical densities at appropriate wave lengths. Specific activities of P^{32} in the nucleotides were then calculated, and after correction for time decay, cellular levels of the nucleotides were computed. In these calculations it was assumed that uniform labeling of all three phosphates in each nucleosidetriphosphate had been achieved. This assumption is verified by the finding that the apparent levels of ATP and GTP did not increase significantly in the control culture during the 5 hour period of the experiment.

The results of two experiments of this type are shown in Table III. The values obtained for the levels of ATP agree well with those determined by the enzymatic analysis and show, once again, that there is no significant alteration in the level of ATP for at least 5 hours after the addition of toxin. The values found for GTP, although differing somewhat in magnitude between the two experi-

ments due to some undetermined error, are consistent within each experiment and show that there is no significant decrease in the GTP level in intoxicated cells. Determination of the kinetics of C^{14}-phenylalanine incorporation showed that toxin brought protein synthesis to a complete halt within 2.5 hours after its addition. Measurement of C^{14}-phenylalanine incorporated into protein was carried out by liquid scintillation counting after the P^{32} had decayed to a low level of activity.

The excellent agreement between the ATP values obtained by two unrelated methods provides convincing evidence that the cellular ATP level does not change significantly for 5 hours in the presence of high levels of toxin. Thus the

TABLE III

ATP and GTP Levels in Intoxicated and Non-Intoxicated HeLa Cells as Determined by Chromatographic Method of Assay

Time	ATP levels*				GTP levels*			
	Exp. I		Exp. II		Exp. I		Exp. II	
	Control	Toxin	Control	Toxin	Control	Toxin	Control	Toxin
hrs.								
0	19.0	—	—	—	5.4	—	—	—
0.5	18.3	19.0	19.8	19.4	5.7	5.4	7.2	6.0
1.5	17.6	19.6	19.6	18.0	5.0	4.8	7.8	7.3
4.0	16.3‡	15.3‡	20.0	19.8	5.1‡	5.2‡	8.2	7.8

* Values expressed as μmoles per gram cell protein.

‡ Samples taken at 5 hours.

possibility that protein synthesis might cease in intoxicated cells because of an effect of toxin on ATP production appears to have been eliminated.

DISCUSSION

In their study on the effect of diphtheria toxin on metabolism of HeLa cells, Strauss and Hendee (1) observed that the kinetics of inhibition of protein synthesis by certain respiratory poisons differed from the kinetics of inhibition by toxin. They showed that, in contrast to a saturating dose of toxin, neither dinitrophenol, which uncouples cytochrome-linked phosphorylation, nor cyanide, which inhibits aerobic respiration, blocks completely the incorporation of amino acids into cell protein (1). In the presence of these inhibitors, uptake of labeled amino acids continues, albeit at a greatly diminished rate. Strauss and Hendee pointed out that if toxin were to act specifically on cytochrome-linked phosphorylation, one might expect some residual protein synthesis from energy provided by the glycolytic process.

The present studies have demonstrated clearly that there is no significant

change in ~P, ATP, GTP, or hexose phosphate contents of HeLa cells over a 5 to 6 hour period following addition of a saturating dose of toxin. Thus injury to the energy metabolism of the cell can be ruled out as a possible primary site of action of diphtheria toxin. Some years ago, Pappenheimer and Williams (18) showed that in the *Cecropia* silkworm, diphtheria toxin exerts a selective action on those tissues that contain a complete cytochrome and succinoxidase system and that of a large number of metabolic poisons tested, only the respiratory inhibitors, cyanide and carbon monoxide under pressure, appeared to act in a manner similar to toxin. Their observations can be reconciled with the recent findings, since those tissues in *Cecropia* with the highest respiratory metabolism may well be those in which protein synthesis is taking place at a maximal rate. Such tissues might therefore be especially sensitive to any toxic agent that acts primarily on protein synthesis.

It will be shown in the following paper (19) that low concentrations of diphtheria toxin block amino acid incorporation into protein in cell-free extracts from mammalian cells supplemented with an excess of ATP and an ATP-generating system.

SUMMARY

Intracellular levels of ATP, GTP, and hexose phosphates have been determined in HeLa cells at intervals after exposure to saturating doses of diphtheria toxin. Toxin causes no significant change in the level of any of these phosphorylated intermediates either in the presence or absence of glucose over a period of at least 5 to 6 hours. It is concluded that the inhibition of protein synthesis which occurs in HeLa cells at about 2 hours after the addition of saturating doses of toxin, does not result from an effect of toxin on energy metabolism.

BIBLIOGRAPHY

1. Strauss, N., and Hendee, E. D., The effect of diphtheria toxin on the metabolism of HeLa cells, *J. Exp. Med.*, 1959, **109,** 145.
2. Kato, I., and Pappenheimer, A. M., Jr., An early effect of diphtheria toxin on the metabolism of mammalian cells growing in culture, *J. Exp. Med.*, 1960, **112,** 329.
3. Strauss, N., The effect of diphtheria toxin on the metabolism of HeLa cells. II. Effect on nucleic acid metabolism, *J. Exp. Med.*, 1960, **112,** 351.
4. Eagle, H., Amino acid metabolism in mammalian cell cultures, *Science*, 1959, **130,** 432.
5. McLimans, W. F., Davis, E. V., Glover, F. L., and Rake, G. W., The submerged culture of mammalian cells: the spinner culture, *J. Immunol.*, 1957, **79,** 428.
6. Yoneda, M., A new culture method designed for kinetic studies on diphtheria toxin production, *Brit. J. Exp. Path.*, 1957, **38,** 190.
7. Raynaud, M., and Relyveld, E. H., La réaction toxine-antitoxine diphtériques, *Ann. Inst. Pasteur*, 1959, **97,** 636.

8. Racker, E., Spectrophotometric measurement of hexokinase and phosphohexokinase activity, *J. Biol. Chem.*, 1947, **167,** 843.
9. Oyama, V. I., and Eagle, H., Measurement of cell growth in tissue culture with a phenol reagent (Folin-Ciocalteau), *Proc. Soc. Exp. Biol. and Med.*, 1956, **91,** 305.
10. Lowry, O. H., Rosebrough, N. J., Fiar, A. L., and Randall, R. J., Protein measurement with the Folin phenol reagent, *J. Biol. Chem.*, 1951, **193,** 265.
11. Barker, J. B., and Summerson, W. H., The colorimetric determination of lactic acid in biological material, *J. Biol. Chem.*, 1941, **138,** 535.
12. Slater, E. C., Spectrophotometric determination of fructose-1:6-diphosphate, hexosemonophosphates, adenosine-triphosphate, and adenosine-diphosphate, *Biochem. J.*, 1952, **53,** 157.
13. Hurlbert, R. B., Schmitz, H., Brumm, A. F., and Potter, V. R., Nucleotide metabolism. II. Chromatographic separation of acid soluble nucleotides, *J. Biol. Chem.*, 1954, **209,** 23.
14. Cohn, W. E., and Bollum, F. J., Chromatography and desalting of nucleotides with ammonium bicarbonate on ion-exchange columns, *Biochem. et Biophysica Acta*, 1961, **48,** 588.
15. Wu, R., Regulatory mechanism in carbohydrate metabolism. V. Limiting factors of glycolysis in HeLa cells, *J. Biol. Chem.*, 1959, **234,** 2806.
16. Eagle, H., Barban, S., Levy, M., and Schulze, H. O., The utilization of carbohydrate by human cell cultures, *J. Biol. Chem.*, 1958, **233,** 551.
17. Keller, E. G., and Zamecnik, P. C., The effect of guanosine diphosphate and triphosphate on the incorporation of labelled amino acids into proteins, *J. Biol. Chem.*, 1956, **221,** 45.
18. Pappenheimer, A. M., Jr., and Williams, C. M., Effects of diphtheria toxin on the cecropia silkworm, *J. Gen. Physiol.*, 1952, **35,** 727.
19. Collier, R. J., and Pappenheimer, A. M., Jr., Studies on the mode of action of diphtheria toxin. II. Inhibition of amino acid incorporation in cell-free systems, *J. Exp. Med.*, 1964, **120,** 1019.