Biochemical Pharmacology, 1962, Vol. 11, pp. 353–370. Pergamon Press Ltd., Printed in Great Britain.

STUDIES RELATED TO THE MODE OF ACTION OF ACTINOMYCIN D*†

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(*Received* 17 *November* 1961; *revised* 29 *December* 1961)

Abstract—(1) The inhibition of growth of *L. arabinosus* and *L. leichmannii* by actinomycin D was prevented by DNA and by 2′-deoxyguanosine, while RNA, a number of purines, pyrimidines, ribonucleosides, deoxyribonucleosides, ribonucleotides, deoxyribonucleotides, amino acids, and vitamins were ineffective. The correlation between the extent of prevention of inhibition and the extent of formation of complexes with actinomycin was good.

(2) Actinomycin D inhibited the incorporation of ^{14}C from [^{14}C]-formate, [1-^{14}C]-glycine, [8-^{14}C]hypoxanthine, [8-^{14}C]adenine, [8-^{14}C]guanine, [2-^{14}C]2,6-diaminopurine, and [6-^{14}C]orotic acid into RNA more than into DNA, and in a number of the experiments the incorporation into guanine was inhibited more than the incorporation into adenine. The biological systems used in these tracer experiments were *L. leichmannii*, Ehrlich ascites tumor cells *in vivo* and *in vitro*, and H.Ep-2 cells in culture and in a non-proliferating system.

(3) Experiments with *L. leichmannii* and H.Ep-2 cells indicated the actinomycin D interfered with the synthesis *de novo* of purine ribonucleotides, while it did not interfere with the formation of ribonucleotides from preformed purines. This interference might be a secondary effect of inhibition of synthesis of RNA.

(4) An attempt has been made to draw together the experimental results of others and those presented here to formulate a hypothesis regarding the mode of action of actinomycin.

ACTINOMYCIN D is an antibiotic that is temporarily effective in the treatment of certain types of experimental and human neoplasms,[1, 2] and a number of investigations have been carried out in efforts to determine its mode of action. In studies with several strains of bacteria that require pantothenate for optimum growth a competiive inhibition of the utilization of pantothenate by actinomycin D was observed.[3] However, pantothenate did not prevent the inhibition of the growth of *Bacillus subtilis*,[4, 5] several species of *Clostridium*,[6] or *Saccharomyces cerevisiae*,[7] did not prevent the antitumor effect of actinomycin D for mouse neoplasms,[8, 9] and did not prevent the cytotoxic effects of actinomycin C for cultured human cells.[10] The inhibition of mycelial

* This work was supported by the Cancer Chemotherapy National Service Center, National Cancer Institute, under the National Institutes of Health Contract No. SA-43-ph-2433, and by grants from the Charles F. Kettering Foundation, the Alfred P. Sloan Foundation, and Grant No. T-13C from the American Cancer Society.

† Abbreviations: RNA, ribonucleic acids; DNA, deoxyribonucleic acids; DPN, diphosphopyridine nucleotide; AMP, adenylic acid; 5′-AMP, adenosine 5′-phosphate; (2′ + 3′)-AMP, mixed 2′ and 3′ isomers of adenylic acid; dAMP, 2′-deoxyadenylic acid; ADP, adenosine diphosphate; ATP, adenosine triphosphate; GMP, guanylic acid; 5′-GMP, guanosine 5′-phosphate; (2′ + 3′)-GMP, mixed 2′ and 3′ isomers of guanylic acid; GTP, guanosine triphosphate; IMP, inosinic acid; UMP, uridylic acid; CMP, cytidylic acid; UTP, uridine triphosphate; CTP, cytidine triphosphate; tris, tris(hydroxymethyl)aminomethane.

‡ Affiliated with Sloan-Kettering Institute for Cancer Research.

growth of *Neurospora crassa* by actinomycins C and D was partially prevented by *p*-aminobenzoic acid, tyrosine, and phenylalanine,[11] and the antimitotic activity of actinomycin C for chick fibroblasts and human cells in culture was completely suppressed by glutathione, although the cytocidal effect was not altered.[12] In studies of the effects of actinomycin I on Ehrlich ascites cells under a variety of conditions, it was found that under appropriate conditions actinomycin I drastically reduced both aerobic and anaerobic glycolysis with little effect on respiration,[13] and other results obtained with *Candida albicans* indicated that this actinomycin might be acting as a phosphate acceptor or might activate the breakdown of ATP.[14] Since actinomycin C caused a decrease in the content of pyridoxal phosphate, but not of pyridoxine, in rat liver, it has also been suggested that this antibiotic affects the conversion of the various free forms of vitamin B_6 into pyridoxal-5-phosphate.[15] The fact that assimilation of ammonia and the formation of inducible enzymes by *B. subtilis* were suppressed by actinomycin C indicates an interference with synthesis of proteins,[16] and in studies with *B. subtilis*,[5] *Staphylococcus aureus*,[17] and cultured cells[18 20] it was found that actinomycins inhibited the synthesis of RNA and of protein more quickly and more extensively than the synthesis of DNA. The interference with the synthesis of RNA is consistent with the observed morphological alterations of the nucleoli of cells by actinomycins.[18–21] On the other hand, DNA prevented the inhibition of growth of *S. aureus*,[17] *B. subtilis*,[22] *Neurospora crassa*[23, 24] and *Streptococcus faecalis*,[24] while RNA was ineffective. Evidence indicative of interaction between DNA and actinomycin was obtained,[17, 22–24] and it was stated that the prevention of inhibition of growth was due to the formation of complexes.[24] Of a number of derivatives of nucleic acids that were tested, 2′-deoxyguanosine was the most effective in preventing the inhibition of growth of *N. crassa* by actinomycin C and also caused the greatest alteration of the spectrum of actinomycin C.[25]

In the present investigation, the effects of actinomycin D upon the synthesis of nucleic acids and precursors of nucleic acids by *Lactobacillus leichmannii*, by Ehrlich ascites tumor cells *in vivo* and *in vitro*, and by human cells in culture and in a buffer solution have been examined. Differences as well as similarities were detected for the various biological species. The possibility of a correlation between ability of nucleic acids and related compounds to prevent inhibition of growth of *Lactobacillus leichmannii* and to form complexes with actinomycin in the bacterial growth medium was also examined with the aid of spectrophotometric measurements. Some of the results that are included in the present report were presented previously in a preliminary report.[26]

METHODS

Experiments with bacteria

Growth and prevention of inhibition. Lactobacillus arabinosus (ATCC 8014) was grown on a chemically defined synthetic medium that was similar to Medium VI of Sauberlich and Baumann[27] with the following exceptions: the quantity of L-cysteine was doubled and a quantity of L-cystine equal to the increased quantity of L-cysteine was added; ammonium chloride, purines, pyrimidines, and folic acid were omitted; one-fifth the quantity of biotin was used, and one-tenth the quantities of the other vitamins were used. *Lactobacillus leichmannii* (ATCC 7830) was grown on Bacto-Vitamin B_{12} Assay Medium (Difco) supplemented with 0·2 mμg of vitamin B_{12} per ml.

The extent of growth was determined turbidimetrically with a Bausch and Lomb Spectronic 20 spectrophotometer, after incubation at 32 °C for 18 hr.

Tests for resistance. In tests for the development of resistance to actinomycin D, cells that had grown in the presence of the drug plus a protective agent were used as inocula for tubes containing graded amounts of actinomycin D, and cells that had never been exposed to the drug were used as inocula for a similar series of tubes; the comparative extents of growth in these two series of tubes indicated the degree of resistance of the previously exposed cells.

Tracer studies. Two 3000-ml Erlenmeyer flasks containing 2000 ml of medium were inoculated with 14 ml of a 1 : 200 dilution of a 24-hr culture of *L. leichmannii* which had been washed with saline and resuspended in a volume of saline that would yield a transmission at 660 mμ of 17 per cent. After incubation for 16 hr and while the cultures were in logarithmic growth, 16 ml of a solution of actinomycin, 100 μg/ml, was added to one flask, and an equal volume of sterile water was added to the other flask. Growth was followed turbidimetrically. Inhibition of growth was evident by 60 min after the addition of the actinomycin, and at this time either 200 μc of [^{14}C]formate (specific activity, 58·4 μc/mg) or 40 μc of [8-^{14}C]hypoxanthine (specific activity, 33·7 μc/mg) was added to each flask, and incubation was continued for 2 hr (this period of time corresponded to approximately one generation period for the uninhibited cells and one-third generation period for the inhibited cells). Growth was stopped by cooling the cultures in ice water, and the cells were separated from the medium by centrifugation in the cold and washed two times with saline. The washed cells were weighed and then suspended in 10 ml of water per g of cells, the suspension was poured into 4 vols. of boiling absolute ethanol, and the mixture was boiled for 5 min. The solid material was removed by centrifugation, and the supernatant fraction was used for two-dimensional chromatography and radioautography, as described elsewhere.[28] The radioactive spots on the chromatograms were assayed for radioactivity with the use of a liquid scintillation counter. The cells were then washed successively two times each with water, cold 5% trichloroacetic acid, water, ethanol, and ether, and the purines of RNA and DNA were isolated and assayed for radioactivity by previously described methods.[29]

Experiments with Ehrlich ascites cells

Tracer experiments in vivo. Swiss mice were implanted intraperitoneally with Ehrlich ascites tumor cells and on the seventh day thereafter were given an intraperitoneal injection of actinomycin D (0·025 mg/kg) 1 hr before, and a similar injection immediately before, injection of sodium [^{14}C]formate (specific activity, 28·6 μc/mg or 58·4 μc/mg) at a dosage of 5–10 μc/25 g, [8-^{14}C]hypoxanthine (specific activity, 35·1 μc/mg) at a dosage of 5·1–5·6 μc/25 g, [8-^{14}C]adenine (specific activity, 20·4 μc/mg) at a dosage of 5·7 μc/25 g, [8-^{14}C]guanine (specific activity, 15·2 μc/mg) at a dosage of 4 μc/25 g, or [2-^{14}C]2 : 6-diaminopurine (specific activity, 3·1 μc/mg) at a dosage of 1·25 μc/25 g. Control animals received an equivalent volume of distilled water instead of actinomycin. Groups of from six to eleven animals were killed 1 hr after the administration of the labeled compound, and the acid-soluble purines and the purines of the RNA and DNA were isolated from the ascites cells and assayed for radioactivity by procedures that have been described previously.[29]

Tracer studies in vitro. Ehrlich ascites tumor cells in ascitic fluid were obtained from Swiss mice 8 days following intraperitoneal implantation of tumor cells. To each of two 10-ml portions of the ascitic cell suspension (approximately $1{\cdot}0 \times 10^8$ cells per ml) was added 2 μc of [8-^{14}C]adenine (specific activity, 10·9 μc/mg), and to one of these 10-ml portions was added actinomycin D to give a concentration of 0·1 μg per ml. A similar experiment in which the concentration of actinomycin D was 2 μg per ml was also run. These suspensions were then incubated under oxygen at 37 °C for 4 hr in a Dubnoff metabolic shaking incubator. Following incubation the cells were separated by centrifugation, and the supernatant fraction was discarded. The cells were suspended in 5 ml of water, and this suspension was poured into 20 ml of boiling absolute ethanol and boiled for 5 min. The undissolved material was separated by centrifugation, and washed successively two times each with water, cold 5% trichloroacetic acid, water, ethanol, and ether. This material was then hydrolysed with 0·3 N sodium hydroxide overnight at 37 °C, and after the hydrolysate was neutralized with 5 N hydrochloric acid, trichloroacetic acid was added to a concentration of 3 per cent to precipitate the DNA and protein, which were then discarded. The solution containing the ribonucleotides was neutralized with 4 N sodium hydroxide and stirred with 2·5 ml of an aqueous suspension of activated carbon (Norite A, 0·4 g/ml). The carbon was separated by centrifugation, washed two times with water, and then treated with 5 ml of ammoniacal ethanol (0·3 ml of concentrated ammonium hydroxide plus 100 ml of 50% aqueous alcohol) to obtain the adsorbed ribonucleotides. The carbon was separated by centrifugation and discarded, and the supernatant material was evaporated to dryness in a stream of dry air. The residue was dissolved in 0·5 ml of water, and 100 μl of this solution was used for electrophoresis on a strip of Whatman no. 3 paper in a 1 M ammonium formate buffer, pH 3·5, at 1000 V for 1½ hr. The AMP and GMP were located by scanning the paper with ultraviolet light, and these areas of paper were cut from the strip and sewed onto sheets of Whatman no. 1 paper and chromatographed one-dimensionally by the ascending technique, using a solvent composed of *n*-butanol, propionic acid, and water [40 : 25 : 35 (by weight)]. The ribonucleotides were again located by scanning with ultraviolet light and eluted with 3 N ammonium hydroxide, and after the concentration of either AMP or GMP was determined spectrophotometrically, known volumes of the solutions were plated on steel planchets for radioassay in a windowless gas-flow counter. The identities of AMP and GMP as mixtures of the 2′- and 3′-isomers were established by chromatographing portions of the above-described alkaline hydrolysate in parallel with known samples of 5′-AMP, (2′ + 3′)-AMP, 5′-GMP, and (2′ + 3′)-GMP in a solvent consisting of saturated ammonium sulfate, *iso*propanol, and 1 M sodium acetate [80 : 2 : 18 (by volume)] and by radioautography of the resulting chromatograms.

Experiments with cells in culture

Experiments with proliferating cells. Human epidermoid carcinoma cells (H.Ep-2)[30] were grown in swirl culture without supplementary gassing in a synthetic medium containing calf serum and containing no purines or pyrimidines.[31] Each swirl flask contained 400, 500, or 800 ml of medium, and while the cells were in logarithmic growth, the respective radioactive substrate and actinomycin D were added (control cultures were run without actinomycin D), and swirling was continued for 24 hr. Cell counts were made by means of a hemacytometer at the time of addition of substrates

and 24 hr later, when the cells were harvested. At the time of harvest the cultures contained approximately $1{\cdot}0 \times 10^6$ cells per ml. The cells were separated by centrifugation, washed two times with saline, and suspended in water in a ratio of 10 ml of water per $2{\cdot}5 \times 10^8$ cells based upon the cell count at the time of harvest. This suspension was poured into 4 vols. of boiling absolute ethanol and boiled for 5 min. The alcoholic extracts were used for chromatography, radioautography, and radioassay in the procedure described above for the experiments with bacteria, and the purines of the RNA and DNA were also isolated and assayed for radioactivity by the procedure described above for experiments with bacteria. In one experiment, in which pyrimidines were isolated and assayed for radioactivity, the combined nucleic acids were obtained by extraction of the acid-treated, de-fatted cells with hot sodium chloride solution and subsequent acidification. The combined nucleic acids were hydrolysed with 72% perchloric acid at 95 °C for 2 hr, and, after the removal of the perchlorate ion as potassium perchlorate, the hydrolysate was chromatographed one-dimensionally on Whatman no. 1 paper by the ascending technique with a solvent composed of *n*-butanol, water, and formic acid [77 : 13 : 10 (by volume)]. The areas of the paper containing the uracil and the thymine were eluted with 3 N ammonium hydroxide, and the eluates were assayed spectrophotometrically and plated on steel planchets for radioassay.

Experiments with cells in buffer

The composition (μmoles per ml) of the buffer solution, which was adjusted to pH 7·5 with 6 N hydrochloric acid, was as follows: tris, 100; magnesium chloride, 3·3; DPN, 0·1; nicotinamide, 40; and fructose diphosphate, 11·4. Cells that had been grown in swirl culture as described above were washed with the buffer solution two times, and then $2{\cdot}5 \times 10^8$ cells were suspended in 10 ml of buffer solution to which had been added the following compounds to give the indicated concentrations (μmoles per ml): [8-^{14}C]adenine (specific activity, 10·9 μc per mg), 0·2; GTP, 0·2; UTP, 0·2; and CTP, 0·2. To a similar suspension of cells, actinomycin D was added to give a concentration of 2 μg per ml. The suspensions were then incubated for 2 hr under oxygen at 37 °C in a Dubnoff shaking incubator. Following the incubation the mixture was transferred to a centrifuge tube, and the beaker was rinsed with 5 ml of medium, which was then added to the mixture in the centrifuge tube. To the resulting mixture, 2 ml of 48% trichloroacetic acid was added, and the precipitate was separated by centrifugation. The precipitate was washed two times with 8% trichloracetic acid and then heated with 5 ml of 0·1 N sodium hydroxide at 80 °C for 45 min. After the pH of the mixture was adjusted to pH 7·0 by the addition of 1·0 N hydrochloric acid, the ribonucleotides were isolated by adsorption on carbon and by electrophoresis on paper, identified by paper chromatography, and assayed for radioactivity by the procedures described above for the experiment with Ehrlich ascites cells *in vitro*.

Determination of alterations of spectra

A Beckman Model DU spectrophotometer was used for the determination of the spectrum of actinomycin D dissolved in the single-strength medium described above for the growth of *L. arabinosus*. The concentration of the actinomycin was 30 μg per ml, and the spectrum was determined over the wavelength range 400–500 mμ; single-strength medium was used as a blank. None of the medium was autoclaved, since

autoclaving caused the medium to become colored. Spectra were also determined for solutions containing 30 μg of actinomycin D and 3·72 μmoles of the test compound per ml, with the exceptions that RNA and DNA were used at concentrations of 660 μg per ml. The test compounds used were purchased from commercial suppliers. The DNA and the RNA were not "highly polymerized", but the same materials were used

TABLE 1. THE EFFECTS OF VARIOUS COMPOUNDS IN PREVENTING THE INHIBITION OF GROWTH OF BACTERIA CAUSED BY ACTINOMYCIN D

Test compound*	Percent growth					
	L. arabinosus			*L. leichmannii*		
	A	B		A	B	
	Without actinomycin D	With actinomycin D†	100 B/A	Without actinomycin D	With actinomycin D‡	100 B/A
None	100	9	9	100	1	1
Adenine	70	14	20	97	20	21
Adenosine	90	11	12	100	13	13
Deoxyadenosine	81	15	19	89	3	3
Adenylic acid	93	3	3	—	—	—
Adenosine diphosphate	97	10	10	—	—	—
Adenosine triphosphate	84	9	11	—	—	—
Deoxyadenylic acid	102	7	7	—	—	—
Guanine	84	10	12	—	—	—
Guanosine	100	9	9	118	39	33
Deoxyguanosine	98	67	68	106	106	100
Guanylic acid	103	13	13	—	—	—
Deoxyguanylic acid	97	13	13	—	—	—
Hypoxanthine	93	13	14	106	9	8
Inosine	93	11	12	106	3	3
Deoxyinosine	87	14	16	114	3	3
Inosinic acid	93	6	6	—	—	—
Inosine diphosphate						
Xanthine	91	6	7	—	—	—
Xanthosine	100	13	13	—	—	—
Thymine	100	5	5	—	—	—
Thymidine	100	5	5	106	2	2
Thymidylic acid	97	4	4	—	—	—
Cytosine	95	5	5	—	—	—
Cytidine	92	5	5	106	1	1
Deoxycytidine	100	5	5	110	1	1
Cytidylic acid	100	6	6	—	—	—
Deoxycytidylic acid	100	8	8	—	—	—
Uracil	104	5	5	—	—	—
Uridine	104	4	4	110	1	1
Deoxyuridine	104	4	4	—	—	—
Uridylic acid	102	5	5	—	—	—
RNA	104	11	11	—	—	—
DNA	100	100	100	—	—	—

* Each test compound was present at a concentration of 5 μmoles per ml except for RNA and DNA which were used at a concentration of 900 μg per ml.

† Actinomycin was present at a concentration of 0·6 μg per ml.

‡ Actinomycin was present at a concentration of 0·05 μg per ml.

for the spectral study as were used for the prevention of inhibition of growth of the bacteria.

RESULTS

Bacteria

In Table 1 are data that show the effectiveness of a number of compounds related to nucleic acids in preventing the inhibition of growth of *L. arabinosus* and *L. leichmannii* by actinomycin D. The data in the A-columns show the effects of the candidate protective agents upon the growth of the bacteria in the absence of actinomycin; the data in the B-columns show the extent of growth in the presence of both the test compound and actinomycin D. These data were combined, as shown in the third column for each bacterial strain, in order to have comparable data that would take into account the effect of the test compound alone upon the extent of growth. These data show that DNA completely protected *L. arabinosus* and that 2′-deoxyguanosine was the next best; 2′-deoxyguanosine completely protected *L. leichmannii* (DNA and RNA were not tested with *L. leichmannii*). With both strains of bacteria, 2′-deoxyguanosine was much more effective than any other compound (except DNA) in preventing growth-inhibition. In addition to the compounds listed in Table 1, a number of vitamins and amino acids were tested, and none was found to prevent the inhibition. Since in studies with other inhibitors it has previously been found that some compounds might stimulate the over-growth of resistant cells in the presence of an inhibitor, rather than truly protect the sensitive cells,[32] cells that grew in the presence of actinomycin D and 2′-deoxyguanosine were tested for resistance to this inhibitor. The data of Table 2 show

TABLE 2. GROWTH OF *L. leichmannii* IN THE PRESENCE OF ACTINOMYCIN D PLUS 2′-DEOXYGUANOSINE AND TESTING OF THE RESULTING CULTURE FOR RESISTANCE TO ACTINOMYCIN D

A. Growth

Tube	Actinomycin D (μg per ml)	2′-Deoxyguanosine (μmoles per ml)	% growth
1	0	0	100
2	0·05	0	0
3	0	5	90
4	0·05	5	90

B. Test for Resistance

Actinomycin D (μg per ml)	% growth	
	Culture from tube 1*	Culture from tube 4*
0	100	100
0·005	103	90
0·01	69	70
0·02	9	8
0·03	0	0
0·04	0	0
0·05	0	0

* This number indicates the tube of A that was used as inoculum in the test for resistance.

that the cells which grew in the presence of actinomycin and 2′-deoxyguanosine were as sensitive to the inhibitor as cells that had not been previously exposed to the inhbitor; therefore, it appears that true protection was afforded by this compound.

To determine if the observed protection was due to inactivation of the actinomycin by the formation of a complex addition product with the protective agent, an attempt was made to correlate spectral data with protective action. Alteration of the spectral absorption curve for actinomycin by the added compound was interpreted as evidence of complexing,[17, 24, 25] and the alterations noted were the change in the optical density of the solution at 440 mμ and the shift in the wavelength of maximum absorption. The resulting data are presented in Table 3. 2′-Deoxyguanosine and DNA, which were

TABLE 3. CORRELATION OF EXTENT OF FORMATION OF COMPLEXES AND EXTENT OF PREVENTION OF INHIBITION OF GROWTH OF *L. arabinosus* BY ACTINOMYCIN D

Test compound	Change of optical density at 440 mμ* (× 1000)	Growth† (% of control)	Change of λ_{max}(mμ)*
None	0	9	0
DNA	−115	100	+10
2′-Deoxyguanosine	−107	68	+10
GMP	− 53	13	+ 3
dAMP	− 51	7	− 2
Guanosine	− 44	9	+ 5
RNA	− 42	11	− 2
ADP	− 42	10	+ 4
2′-Deoxyadenosine	− 33	19	+ 5
AMP	− 27	3	+ 2
ATP	− 20	11	0
2′-Deoxyinosine	− 19	16	+ 5
Adenine	− 14	20	+ 1
Inosine	− 7	12	0
2′-Deoxycytidine	− 2	5	+ 1
CMP	− 2	6	0
Adenosine	− 1	12	+ 4
Cytidine	0	5	0
Xanthosine	+ 3	13	0
IMP	+ 5	6	0
Uridine	+ 5	4	0
Hypoxanthine	+ 7	14	+ 1
UMP	+ 8	5	0
Cytosine	+ 8	5	0
Uracil	+ 28	5	0
2′-Deoxyuridine	+ 28	4	+ 2
Thymine	+ 38	5	+ 1
Thymidine	+ 38	5	0

* Spectral studies were performed on complete growth medium containing 30 μg of actinomycin D and 3·72 μmoles of the test compound per ml, with the exceptions that RNA and DNA were used at a concentration of 660 μg per ml.

† For the growth studies, actinomycin D was used at a concentration of 0·6 μg per ml, and the test compounds were used at a concentration of 5 μmoles per ml, except for RNA and DNA, which were used at a concentration of 900 μg per ml.

much more effective than the other compounds in preventing the inhibition of actinomycin D, caused greater alterations of the spectrum of actinomycin than the other compounds. These data indicate that there is some correlation between the extent of complex formation and the effectiveness in preventing inhibition. These results are in general agreement with those of Kersten[25] and serve as an extension of those results,

since in the present experiments the extent of complex formation in the complete growth medium was determined.

The data of Table 4 show that, in *L. leichmanni*, antinomycin D interfered with the incorporation of ^{14}C from [^{14}C]formate into the purines of both RNA and DNA and with the incorporation of ^{14}C from [8-^{14}C]hypoxanthine into the purines of RNA, but

TABLE 4. EFFECTS OF ACTINOMYCIN D UPON THE INCORPORATION OF RADIOACTIVE PRECURSORS INTO THE PURINES OF THE NUCLEIC ACIDS OF *L. leichmannii*

Radioactive precursor	Specific activity of inhibited as percent of control			
	RNA		DNA	
	Adenine	Guanine	Adenine	Guanine
[^{14}C]Formate	2	3	10	16
[8-^{14}C]Hypoxanthine	14	13	76	117

Actinomycin D was added to exponentially growing bacteria to a concentration of 0·79 μg per ml, and 1 hour later [^{14}C]formate was added to give a concentration of 0·085 μc per ml or [8-^{14}C]hypoxanthine was added to give a concentration of 0·017 μc per ml. Sterile water instead of actinomycin solution was added to the control cultures. The cells were harvested 2 hr following the addition of the radioactive substrates.

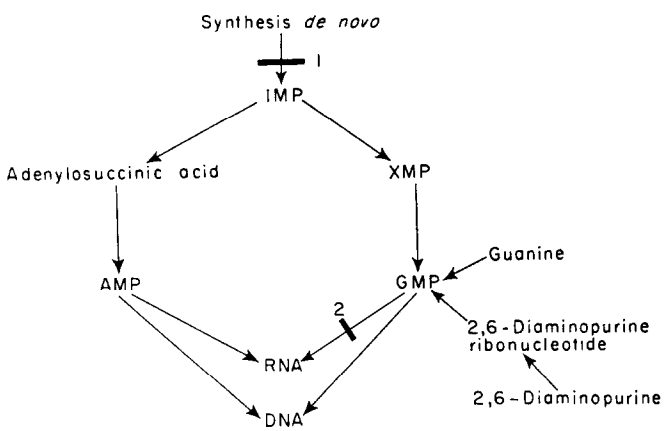

FIG. 1. Possible sites of action of actinomycin D. The numbered cross-bars indicate possible sites of action of the inhibitor.

not into the purines of DNA. These results indicate that the inhibitor interferes with (a) the *de novo*-synthesis of purines (site 1 of Fig. 1) and (b) the conversion of ribonucleotides to RNA. If ribonucleotides are precursors of the deoxyribonucleotides, which eventually are incorporated into DNA, the data suggest there was probably no

interference with the formation of ribonucleotides from [^{14}C]hypoxanthine, but there was interference with the incorporation of these ribonucleotides into RNA. On the other hand, since the incorporation of ^{14}C from [^{14}C]formate into the purines of DNA was inhibited, there was evidently interference with the *de novo*-synthesis of purine ribonucleotides.

TABLE 5. EFFECTS OF ACTINOMYCIN D UPON THE DISTRIBUTION OF ^{14}C FROM [^{14}C]-FORMATE AND FROM [8-^{14}C]HYPOXANTHINE AMONG COMPONENTS OF EXTRACTS OF *L. leichmannii*

Compound	Percent of total radioactivity			
	[^{14}C]Formate		[8-^{14}C]Hypoxanthine	
	Control	Treated*	Control	Treated*
Formylglycinamide ribonucleoside†	31	14	—	—
Serine	3	6	—	—
Glutamine	2	5	—	—
Hypoxanthine	3	8	0	0·2
Inosinic acid	7	21	—	—
Guanine†	—	—	0	0·4
Guanylic acid	2	8	0	0·4
Adenine	11	26	2	2·0
Adenosine	3	0	4	2·0
Adenylic acid	8	0	7	8·0
Adenosine diphosphate	3	2	4	5·0
Adenosine triphosphate	—	—	2	2·0
DPN	10	6	68	68·0
Unknown 1	1	0	6	5·0
Unknown 2	—	—	7	5·0
Unknown 3	17	4	—	—
Unknown 4	—	—	0	0·4
Unknown 5	—	—	0	0·5
Unknown 6	—	—	0	0·1
Unknown 7	—	—	1	0·5
Total activity (counts/min)	$4·38 \times 10^3$	$1·90 \times 10^3$	$6·92 \times 10^3$	$3·30 \times 10^4$

* Actinomycin was present at a concentration of 0·79 μg per ml.
† Tentative identification of this material.

Table 5 contains data that show the distribution of radioactivity from [^{14}C]formate and from [8-^{14}C]hypoxanthine in the extracts of the same cells that were used to obtain the data of Table 4. Actinomycin D decreased the fixation of ^{14}C from [^{14}C]formate into soluble components of the cells and caused some alteration in the distribution of the radioactivity. The actinomycin caused an increase in the quantity of ^{14}C from [8-^{14}C]hypoxanthine fixed into the soluble fraction of the cells, but did not alter the distribution of the radioactivity among the components of the soluble fraction.

Ehrlich ascites cells

Table 6 contains data that show the effects of actinomycin D upon the *in vivo*-incorporation of ^{14}C from various precursors into the acid-soluble purines and the purines of RNA and DNA of Ehrlich ascites tumor cells.

In the experiments with [^{14}C]formate, [8-^{14}C]hypoxanthine, and [8-^{14}C]adenine as substrates, the specific activities of the guanine of the soluble fraction and the guanine of the DNA were affected to approximately the same extent, and therefore a single

metabolic block might account for both inhibitions. Actinomycin caused a greater decrease in the specific activity of the guanine of the RNA than of the guanine of the soluble fraction and of the DNA and prevented the entrance of ^{14}C into the guanine of RNA more than it prevented the entrance of ^{14}C into the adenine of RNA. These results might be explained by assuming that a metabolic blockade occurs at site 2 of Fig. 1. Since the inhibitor was injected into the animals prior to the injection of the

TABLE 6. EFFECTS OF ACTINOMYCIN UPON THE INCORPORATION OF RADIOACTIVE PRECURSORS INTO ACID-SOLUBLE AND NUCLEIC-ACID PURINES OF EHRLICH ASCITES CELLS *in vivo*

Exp. No.	Precursor	Specific activity as % of controls					
		Adenine			Guanine		
		Acid-sol.	RNA	DNA	Acid-sol.	RNA	DNA
1	[^{14}C]Formate	106	77	79	40	20	33
2	[^{14}C]Formate	69	51	73	38	—	53
3	[^{14}C]Formate	69	67	117	36	25	53
4	[^{14}C]Formate	72	81	91	—	25	48
5	[^{14}C]Formate	73	63	70	32	14	32
6	[8-^{14}C]Hypoxanthine	81	77	72	38	18	34
7	[8-^{14}C]Hypoxanthine	67	59	67	41	19	39
8	[8-^{14}C]Adenine	72	49	80	33	14	44
9	[8-^{14}C]Guanine	126	39	55	76	43	38
10	[2-^{14}C]2:6-Diaminopurine	—	—	—	96	38	54

Note: Actinomycin was administered at a dosage of 0·025 mg per kg 1 hr before and immediately before the administration of the radioactive compound, and the mice were sacrificed 1 hr following the administration of the radioactive compound. From six to eleven mice were used in each control or each treated group.

radioactive compound, it is possible that the blockade at site 2 could cause increases in the sizes of the pools of guanine ribonucleotides, which would result in dilution of the subsequently formed radioactive guanine compounds. Thus the specific activities of the guanine of the soluble fraction and the guanine of the DNA would be decreased to about the same extent. The observed smaller decreases of the specific activities of the adenine of the three fractions might be secondary effects of the blockade at site 2.

The results obtained in the experiments with [8-^{14}C]guanine and [2-^{14}C]2 : 6-diaminopurine are similar to those obtained with the other substrates, but there was less difference in the effects upon incorporation of ^{14}C into the guanine of RNA and the guanine of DNA. The higher specific activities of the soluble fractions might be due to the presence of free bases derived from the substrates.

A comparison of the data obtained with [^{14}C]formate and those obtained with [8-^{14}C]hypoxanthine leads to the conclusion that there was no interference with synthesis of the purine moiety *de novo*. As actinomycin inhibits the production and survival of Ehrlich ascites cells *in vivo*,[2] it is evident that interference with synthesis *de novo* is not essential for the anticancer activity of this agent.

The data of Table 7 show that the synthesis of RNA *in vitro* was also inhibited by actinomycin D.

H. Ep-2 cells in culture

The data of Table 8 for [^{14}C]formate, [1-^{14}C]glycine, and [8-^{14}C]hypoxanthine show that actinomycin interfered with the synthesis of purines *de novo* (site 1, Fig. 1) and with the formation of RNA. There was also a greater inhibition of incorporation of

TABLE 7. EFFECTS OF ACTINOMYCIN D UPON THE *in vitro*-INCORPORATION OF ^{14}C OF [8-^{14}C]ADENINE INTO THE RIBONUCLEIC ACID OF EHRLICH ASCITES CELLS*

Exp. no.	Concentration of actinomycin D (μg per ml)	Number of cells per ml	Specific activity as % of control	
			(2′ 3′)AMP	(2′ 3′)GMP
1	0	107,500,000	100	100
	0·1	107,500,000	73	69
2	0	111,250,000	100	100
	2·0	111,250,000	44	<35†

* To 10 ml of ascitic fluid and cells obtained from mice bearing Ehrlich ascitic tumor was added 2 μc of [8-^{14}C]adenine, and the suspension was incubated under oxygen at 37°C for 4 hr in a Dubnoff metabolic shaking incubator.

† Although the exact value for the activity of this (2′ + 3′)GMP could not be determinded because of the low level of activity, it was known to be less than the indicated value.

TABLE 8. EFFECTS OF ACTINOMYCIN D UPON THE INCORPORATION OF ^{14}C OF RADIOACTIVE SUBSTRATES INTO THE PURINES OF NUCLEIC ACIDS OF H.Ep-2 CELLS IN CULTURE*

Exp. no.	Radioactive substrate	Specific activity as % of controls			
		RNA		DNA	
		Ad	Gu	Ad	Gu
1	[^{14}C]Formate	19	8	39	31
2	[^{14}C]Formate	15	6	39	27
3	[^{14}C]Formate	24	10	39	34
4	[^{14}C]Formate	38	23	67	53
5	[1-^{14}C]Glycine	23	10	37	35
6	[8-^{14}C]Hypoxanthine	48	20	147	113
7	[8-^{14}C]Hypoxanthine	48	27	98	104
8	[8-^{14}C]Hypoxanthine	56	38	121	151
9	[8-^{14}C]Adenine	53	42	153	177
10	[8-^{14}C]Adenine	52	49	117	163
11	[8-^{14}C]Guanine	246	35	322	162
12	[2-^{14}C]2:6-Diaminopurine	100	38	156	113
13	[6-^{14}C]Orotic acid	(uracil: 70)		(thymine: 116)	

* Actinomycin was present at a concentration of 0·05 μg/ml, and the period of growth in the presence of the inhibitor and the radioactive substrate was 24 hr.

^{14}C into RNA-guanine than into RNA-adenine. It is possible that the extent of synthesis of RNA is limited by the availability of guanine-containing intermediates, and thus the entry of adenine into the RNA is also limited. No specific inhibition of incorporation of ^{14}C into DNA-guanine occurred. The difference in effect upon RNA-guanine and DNA-guanine is consistent with the possibility that there is an

intermediate (possibly GDP) that is common to both RNA and DNA and that actinomycin interferes with the utilization of this intermediate for the synthesis of RNA, but does not inhibit the utilization of the intermediate for synthesis of DNA.

The existence of site 2 was further substantiated by the results obtained with [8-^{14}C]guanine, [2-^{14}C]2 : 6-diaminopurine, and [8-^{14}C]adenine. The incorporation of each of these precursors would be independent of site 1, and the data for each substrate show a preferential inhibition of the synthesis of RNA. The increased incorporation into the DNA might be the result of the diversion of the nucleotides from the synthesis of RNA to that of DNA, as a result of the blockade at site 2. This diversion might also be the cause of the increase in conversion of the [^{14}C]guanine moiety and the [^{14}C]2 : 6-diaminopurine moiety into the [^{14}C]adenine moiety, with subsequent incorporation into the RNA and DNA. However, it is interesting that the incorporation of [8-^{14}C]adenine into the RNA was inhibited and the pattern obtained with this substrate was very similar to that obtained with [8-^{14}C]hypoxanthine.

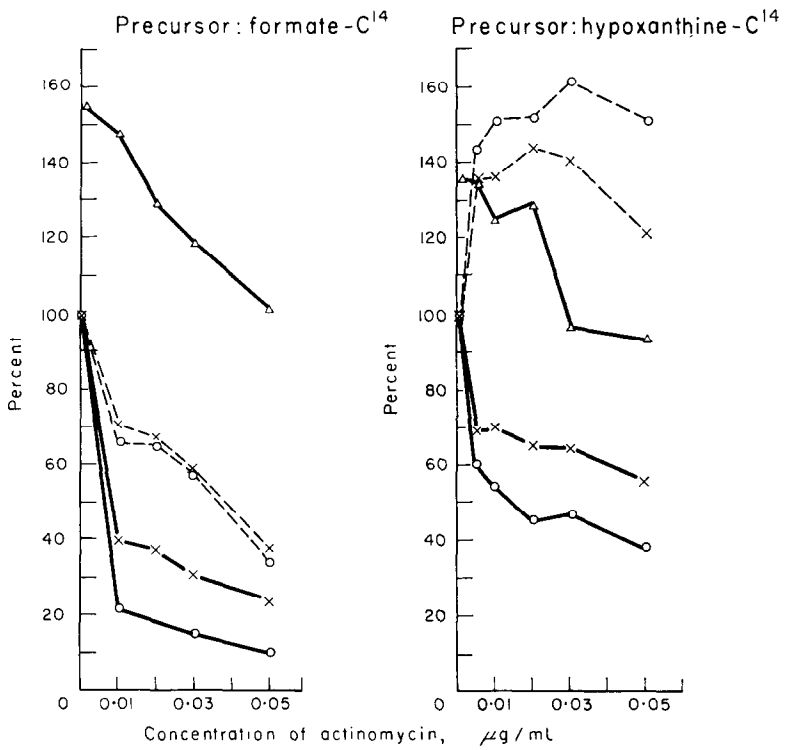

FIG. 2. Effects of actinomycin D upon the increase in cell count of H.Ep-2 cells and upon the incorporation of radioactive precursors into the nucleic acids of these cells. The period of growth in the presence of the radioactive substrate was 24 hr. ×———×, specific activity of RNA-adenine (per cent of control); ×- - - -×, specific activity of DNA-adenine (per cent of control); ○———○, specific activity of RNA-guanine (per cent of control); ○- - - -○, specific activity of DNA-guanine (per cent of control); △———△, cell count (per cent of initial count).

The data obtained with [6-^{14}C]orotic acid as substrate also show a slight inhibition of synthesis of RNA without inhibition of synthesis of DNA. These results are consistent with those obtained by others with tritiated uridine and thymidine.[33]

The graphs in Fig. 2 show the effects of increasing concentrations of actinomycin D upon the increase of cell count of cultures of H.Ep-2 and upon the incorporation of ^{14}C from [^{14}C]formate and from [8-^{14}C]hypoxanthine into the purines of the nucleic acids. It appears that, at a concentration of 0·01 μg per ml, the drug has a greater effect upon the synthesis of RNA than it does upon the survival and multiplication of cells during the time interval studied. Increasing the concentration beyond this point had relatively little effect upon the incorporation into the nucleic acids, but greatly reduced or completely prevented the increase in cell count. This fact suggests that the primary site of action of actinomycin might be outside the area of nucleic acid synthesis or that

TABLE 9. EFFECTS OF ACTINOMYCIN D ON THE DISTRIBUTION OF ^{14}C FROM [^{14}C]FORMATE AMONG COMPONENTS OF EXTRACTS OF H.Ep-2 CELLS

	Concentration of actinomycin D				
	0·0 μg/ml	0·01 μg/ml	0·02 μg/ml	0·03 μg/ml	0·05 μg/ml
	% of total radioactivity detected on the chromatogram				
Serine	2	2	2	3	5
Formylglycinamide ribonucleoside*	1	1	1	1	1
Cysteine	1	1	1	1	1
Methionine sulfoxide	1	1	1	1	1
Hypoxanthine	25	24	24	23	19
Xanthine	—	—	—	—	—
Adenine	3	4	3	3	3
Inosine	13	15	15	15	12
Adenosine	—	—	—	3	3
Xanthosine	1	—	—	1	—
IMP	1	1	1	1	1
AMP	26	25	27	27	29
ADP	9	9	9	6	8
ATP	3	3	3	2	3
GMP	5	4	4	4	5
DPN	11	10	11	11	10
Unknown 51	1	1	1	1	1
Unknown 53	1	1	1	1	1
	Total activity (counts/sec)				
	786	685	879	835	709
	Total activity (% of control)				
	100	87	112	106	90

* Tentative identification.

the slightly greater effects upon nucleic acid synthesis obtained with the higher concentrations of the drug are sufficient to cause a great decrease in the viability of the cells. The increase in specific activity of the DNA-purines with [^{14}C]hypoxanthine as substrate, which occurred in the absence of an increase in cell count, might be due to an increase in the content of DNA per cell.

Tables 9 and 10 contain data for the distribution of ^{14}C among the components of extracts of the same cells that were used to obtain the data of Fig. 2. The data of Table 9 show that actinomycin did not alter the distribution of ^{14}C from [^{14}C]formate among the various compounds, and likewise it did not significantly alter the total

quantity of ^{14}C that was fixed into the soluble compounds. This was true even at a concentration (0·05 μg/ml) that prevented an increase in the cell count. The data of Table 10 show that with [^{14}C]hypoxanthine as the substrate there was also no alteration of the distribution of ^{14}C among the compounds, but with this precursor the total quantity of ^{14}C that was present in the extract increased with increasing concentration of actinomycin. These results would be consistent with the possibility that actinomycin

TABLE 10. EFFECTS OF ACTINOMYCIN D ON THE DISTRIBUTION OF ^{14}C FROM [8-^{14}C]-HYPOXANTHINE AMONG COMPONENTS OF EXTRACTS OF H.Ep-2 CELLS

	Concentration of actinomycin D					
	0·0 μg/ml	0·005 μg/ml	0·01 μg/ml	0·02 μg/ml	0·03 μg/ml	0·05 μg/ml
	% of total radioactivity detected on the chromatogram					
Hypoxanthine	9	10	9	11	8	10
Xanthine	1	1	1	1	1	1
Inosine	4	5	6	6	4	5
Adenosine	4	3	2	3	3	2
IMP	1	1	1	1	1	1
AMP	14	21	20	22	24	21
ADP	22	22	24	21	27	27
ATP	16	12	13	12	12	14
GMP	1	1	1	1	1	1
DPN	22	19	20	18	16	16
Unknown 51	2	2	1	1	2	2
Unknown 53	4	3	3	3	2	1
	Total activity (counts/sec)					
	171	358	370	431	468	554
	Total activity (% of control)					
	100	209	216	252	274	324

interferes at a site such as 2 and causes an accumulation of some unidentified intermediate which, in turn, would stop by a negative feedback mechanism *de novo*-synthesis at some point prior to the formation of IMP, but would not interfere with the incorporation of [8-^{14}C]-hypoxanthine into soluble intermediates and into the DNA. This possibility suggests that the interference with *de novo*-synthesis is a secondary rather than a primary site of action of actinomycin, and as reported above, no interference with *de novo*-synthesis was observed with the Ehrlich ascites cells.

The data of Table 11 show that actinomycin inhibited the incorporation of [8-^{14}C]-adenine into the RNA of H.Ep-2 cells during incubation in a medium that was deficient in most of the compounds that are required for the growth and multiplication of the cells. This result is consistent with the possibility that the interference with the synthesis of RNA is a direct effect of the drug, rather than an indirect effect resulting from the general slowing down or stopping of cellular proliferation.

DISCUSSION

Similarities and differences in the effects of actinomycin are apparent in the various biological systems used. However, all of the results obtained can be rationalized by the postulation of two metabolic blocks, one on synthesis of purine nucleotides

de novo and one on the utilization of guanine nucleotides for synthesis of RNA (sites 1 and 2, Fig. 2). Since site 2, but not site 1, appears to be common to all of the systems studied, it may be postulated that it is at site 2 that actinomycin acts to inhibit growth. This block is consistent with the evidence obtained by other techniques in other systems[5, 17–20] that actinomycin inhibits the synthesis of RNA more than that of DNA. Inhibition at site 2, rather than a general block of RNA polymerase, is also consistent with the observation that actinomycin in general inhibited the incorporation

TABLE 11. EFFECT OF ACTINOMYCIN D UPON THE INCORPORATION OF ^{14}C FROM [8-^{14}C]ADENINE INTO THE RNA OF H.Ep-2 CELLS INCUBATED IN A BUFFER SOLUTION*

Exp. no.	Concentration of actinomycin D, (μg per ml)	Specific activity of (2′ + 3′)-AMP, as % of control
1	0	100
	2	23
2	0	100
	2	25

* Washed cells were incubated in a buffer solution containing the following (μmoles per ml):tris, 100; magnesium chloride, 3·3; DPN, 0·1; nicotinamide, 40; fructose diphosphate, 11·4; GTP, 0·2; UTP, 0·2; CTP, 0·2; and adenine-8-^{14}C (specific activity, 10·9 μc per mg), 0·2. The period of incubation under oxygen at 37° in a Dubnoff shaking incubator was 2 hr.

of precursors into guanine of RNA more than into adenine of RNA. The observed effects on the incorporation of some precursors into polynucleotide adenine, as well as the block of synthesis *de novo* observed in some systems, could well be effects secondary to a block at site 2.

The possible role of complex formation in the inhibitory action of actinomycin is still not well defined. It is interesting that although there is evidence of greater binding of actinomycin by DNA than by RNA[17, 22–24] and although the inhibitory effect of actinomycin for micro-organisms is prevented to a much greater extent by DNA than by RNA, one of the most consistently observed effects of the drug has been the greater inhibition of the synthesis of RNA than that of DNA. This is contrary to the observations that the drug inhibited the DNA polymerase, but not the polynucleotide phosphorylase, system *in vitro*,[17] and inhibited the multiplication of a DNA virus, but not a RNA virus, in cultured cells.[33] The data presented in the present report show that the nature of the purine moiety, as well as the nature of the sugar moiety, influences the extent of complex formation, and one might speculate that deoxyribonucleic acids of different base contents might complex to different extents. One might also speculate that the more extensive complexing with guanine compounds observed in the present study might be related in some way to the inhibition of the incorporation of guanine into RNA that was observed for all of the systems studied. In all of the experiments

related to the detection of complexes, much larger quantities of the drug were used than the quantities required for the toxic effects, and therefore caution should be exercised in interpreting the significance of the data.

It is possible that the very small quantities of actinomycin which inhibit growth may be combined with certain specific DNA molecules in certain cellular loci, while other DNA molecules are not affected. In the light of existing observations, one might suggest that the actinomycin is preferentially fixed by the DNA of the nucleoli, and morphological alteration of the nucleoli results. The actinomycin-bound DNA could then no longer function as a template for the synthesis of RNA; accordingly, the extent of synthesis of RNA and of protein would decrease. In the meantime, however, the bulk of the nuclear DNA would not be affected by the small quantity of the drug, and hence its normal functions, including its role in the synthesis of new DNA, could continue. This unbalanced growth might result in the formation of cells with high contents of DNA and eventually in the death of the cells. The suggested dissociation of the DNA-actinomycin complex[17] might also account for the recovery of cells that have been exposed to low concentrations of actinomycin, such as was observed in the course of the present study.

Acknowledgements—The authors gratefully acknowledge the technical assistance of the following persons: Dr. George G. Kelley, Miss Doris Adamson, Mrs. Ellen Horton, and Mrs. Sue Vail for providing the cultured cells; Miss Linda Simpson, Miss Tommie Lou Barker, and Miss Jane Golden for experiments with Ehrlich ascites cells; Mrs. Jo Ann Alexander, Mrs. Ann Dodson, and Mrs. Susan Briggs for chemical isolations, chromatography, and radioautography; Miss Bonnie Bowdon for bacteriological experiments: and Mrs. Jane Hazelrig for radioassays.

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