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SENSITIVITY OF *TRYPANOSOMA EQUIPERDUM* TO THE ACTION OF TUMOR-INHIBITORY ANTIBIOTICS *IN VITRO**

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Abstract—*Trypanosoma equiperdum*, a member of the *brucei–evansi* group of African trypanosomes, is sensitive to the following tumor-inhibitory antibiotics: actinomycin D, mitomycin C, porfiromycin, phleomycin, and pactamycin. Only pactamycin inhibited trypanosomal carbohydrate metabolism *in vitro*, an action that might have contributed to the ability of this antibiotic to depress protein synthesis and various pathways of nucleic acid metabolism. The inhibitory effects of the other antibiotics were restricted to trypanosomal nucleic acid metabolism.

Actinomycin D, $1{\cdot}6 \times 10^{-8}$ M, inhibited the incorporation of ^{3}H-adenine and ^{3}H-uracil into RNA by approximately 50%, but had no effect at this concentration upon the incorporation of derivatives of these isotopes into DNA. Higher concentrations had no effect upon the incorporation of ^{3}H-adenine and ^{3}H-thymidine into DNA, but did inhibit the incorporation of derivatives of ^{3}H-uracil into DNA. The distribution of radioactivity in the nucleotide components of the acid-soluble fractions suggested that the predominant action of actinomycin D was to inhibit RNA polymerase.

Mitomycin C and its methyl congener, porfiromycin, $2{\cdot}2 \times 10^{-4}$ M and $5{\cdot}7 \times 10^{-4}$ M, respectively, inhibited the incorporation of derivatives of ^{3}H-adenine and ^{3}H-uracil into RNA and DNA by approximately 50%. At $7{\cdot}5 \times 10^{-6}$ M and $1{\cdot}1 \times 10^{-5}$ M, mitomycin C and porfiromycin inhibited the incorporation of ^{3}H-thymidine into DNA by approximately 50%, and at $7{\cdot}5 \times 10^{-5}$ M and $7{\cdot}2 \times 10^{-5}$ M, the evolution of $^{14}CO_2$ derived from orotic acid-^{14}COOH. The ability of the mitosane antibiotics, at relatively low concentrations, to inhibit the incorporation of thymidine into DNA suggested that interference with DNA replication was a prominent feature of this group. The data also indicated that at higher concentrations, mitomycin C and porfiromycin inhibited RNA polymerase—whether by direct action or as a consequence of their effects upon DNA synthesis remains unsettled.

Phleomycin, $1{\cdot}3 \times 10^{-4}$ M, inhibited the incorporation of ^{3}H-adenine and ^{3}H-uracil into RNA by approximately 50%. At this concentration, phleomycin inhibited the incorporation of derivatives of ^{3}H-uracil into DNA to a greater extent than the derivatives of ^{3}H-adenine. At $3{\cdot}2 \times 10^{-6}$ M, phleomycin inhibited the incorporation of ^{3}H-thymidine into DNA by approximately 50%. The action of phleomycin seemed to be qualitatively similar to that of the mitosane antibiotics, except for its inability to inhibit orotidylic acid decarboxylase.

ATTENTION has been directed to the usefulness of various protozoan test systems for the detection and investigation of antitumor agents.[1–7] With regard to tumor-inhibitory antibiotics, actidione, actinoleukin, and actinomycins D, F, and P_2 are potent inhibitors of the growth of *Tetrahymena pyriformis*, *Ochromonas malhamensis*, and *Crithidia fasciculata*, while L-azaserine was found to be trypanocidal against

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Trypanosoma equiperdum.[8] Data are presented herein to show that *T. equiperdum*, a member of the *brucei–evansi* group of African trypanosomes, is also sensitive to the following tumor-inhibitory antibiotics: actinomycin D, mitomycin C, porfiromycin, phleomycin, and pactamycin. At concentrations that had no effect upon trypanosomal carbohydrate metabolism or protein synthesis *in vitro*, all save pactamycin inhibited various pathways of pyrimidine and purine metabolism. The primary inhibitory action of pactamycin seemed to be on carbohydrate metabolism, causing as a consequence generalized depression of other metabolic processes.

MATERIALS AND METHODS

Orotic acid-^{14}COOH, ^{3}H-uracil, ^{3}H-thymidine, ^{3}H-adenine, and phenylalanine-U-^{14}C were obtained from the New England Nuclear Corp.

Generous donations of phleomycin were made by the Bristol Laboratories; porfiromycin and pactamycin by the Upjohn Co. Actinomycin D was kindly given by Dr. J. Bryant, Department of Medicine, University of Vermont College of Medicine. Mitomycin C was obtained from Nutritional Biochemicals Corp.

T. equiperdum was maintained in 8- to 10-week-old Ha/ICR albino female mice by periodic intraperitoneal injections of blood from an infected donor into fresh recipients. When mice were thus inoculated with 10^6 trypanosomes (determined by hemocytometer count), parasitemia levels 48 hr later averaged about 10^9 per ml. Sufficient trypanosomes for isotope uptake studies *in vitro* were obtained by collecting blood from several ether-anesthetized mice after severing the right axillary arteries, adding a small amount of heparin to prevent coagulation, and separating the trypanosomes from other blood elements by differential centrifugation. This procedure was followed: 2–3 ml of heparinized blood were placed in a 12-ml graduated glass centrifuge tube, chilled in an ice bath, and diluted to 8 ml with cold, calcium-free phosphate buffer medium (pH 7·6; 0·116 M) containing 0·01 M glucose and 2% fraction V bovine serum albumin.[9] After gentle but thorough mixing, the suspension was centrifuged at 1,700 *g* for 10 min, and the trypanosomes formed a whitish layer between the supernatant fluid and the packed red cells. The clear supernatant was removed by aspiration and was replaced by 7–8 ml of fresh medium, delivered carefully to avoid disturbing the layers beneath. The trypanosomal layer was then gently resuspended with an applicator stick, and the suspension was aspirated and placed in a clean receptacle. Contamination with blood cells was generally less than 1%.

For whole-cell incubation studies, 10^8 vigorously motile trypanosomes, suspended in the previously described buffer medium, were placed in either 25-ml Erlenmeyer flasks or 15-ml capacity Warburg vessels (depending upon the nature of the reaction to be studied). Vessels contained uniform volumes (usually 0·1 ml) of graded concentrations of particular antibiotics in solution or equivalent volumes of vehicle alone to serve as controls. The volume in each reaction vessel at this point was 2·9 ml. After the vessels were equilibrated in air for 15 min at 37° in a Dubnoff shaking incubator, 0·1-ml aliquots of radioactive substrate were introduced, and incubation was continued for an additional 60 min.

Except that the phospholipid fraction was not removed, standard Schmidt and Thannhauser[10] and Schneider[11] procedures were followed to determine the extent of incorporation of radioactivity derived from isotopic substrates into acid-soluble and protein fractions, RNA and DNA. When isotopic pyrimidines or adenine were

introduced as substrates, their radioactive mono-, di-, and triphosphonucleotide derivatives in the acid-soluble fractions were separated by means of ECTEOLA-cellulose ion-exchange column (0·8 × 10 cm) chromatography, by the technique of discontinuous elution with appropriate concentrations of HCl.[12, 13] Separation was facilitated by the admixture of nonradioactive counterparts which served as carriers of the minute amounts of radioactive mono-, di-, and triphosphonucleotides present in the acid-soluble extracts. The columns were encased in an ice-bath to minimize the risk of autolytic cleavage of nucleotide triphosphates to mono- and diphosphates during chromatography. Individual fractions were monitored at 260 mμ with a Vanguard ultraviolet analyzer connected to a Rinco fraction collector. Radioactivity was measured by liquid scintillation counting (Packard Tri-Carb scintillation spectrometer, model 3002) of 0·2-ml aliquots dissolved in a 10-ml toluene:absolute ethanol (14:5) mixture containing 0·23 % of 2,5-diphenyloxazole (PPO). Samples were checked for quenching by addition of internal standards.

To measure evolution of $^{14}CO_2$, Warburg vessels were used which contained 0·3 ml 2 N NaOH in the center wells to trap liberated gas. The vessels were tightly capped with rubber stoppers of the type used on serum bottles. At the end of the incubation, 0·3 ml 6 N $HClO_4$ was injected by hypodermic needle and syringe through the rubber stoppers into the reaction mixtures. After allowing 15 min for complete absorption of $^{14}CO_2$, the stoppers were removed, and 0·1-ml aliquots of the NaOH solution were withdrawn for assay of radioactivity by the method described above.

The glucostat method[14, 15] was used to measure the amount of glucose remaining in the medium after 10^8 trypanosomes were incubated in the presence of antitumor antibiotics for 60 min at 37°. The amount of pyruvate excreted into the medium by the end of the incubation period was measured by the method of Koepsell and Sharpe.[16]

RESULTS

Effects on carbohydrate metabolism in vitro

It has been established that the motility and cellular integrity of the *brucei–evansi* group of trypanosomes, of which *T. equiperdum* is a member, depend upon a supply of exogenous glucose (or other utilizable carbohydrate) and an efficiently operating Emden-Meyerhof-Parnas glycolytic pathway.[17, 18] Therefore it is necessary, before ascribing antitrypanosomal action of a chemical agent to production of specific biochemical lesions in other areas of metabolism, to determine whether such an agent inhibits trypanosomal carbohydrate metabolism which could result in generalized, nonspecific depression of all metabolic processes.

The data in Table 1 indicate that actinomycin D, mitomycin C, porfiromycin, and phleomycin, at the highest concentrations used in other studies to be described later, had no significant effect *in vitro* on either the consumption of glucose or the production of pyruvate by *T. equiperdum*. The values obtained for normal glucose consumption and pyruvate production, namely, approximately 10 μmoles of glucose consumed and 18 μmoles of pyruvate produced by 10^8 trypanosomes incubated for 1 hr at 37°, are similar to those reported by others.[18–21] Pactamycin, on the other hand, inhibited both glucose consumption and pyruvate production at concentrations used in other studies described below. Since it was previously found,[20, 21] as we have confirmed, that approximately 1·8 moles of pyruvate are excreted by *T. equiperdum* for each mole

TABLE 1. EFFECT OF TUMOR-INHIBITORY ANTIBIOTICS UPON GLUCOSE CONSUMPTION AND PYRUVATE PRODUCTION BY *T. equiperdum* IN VITRO*

Antibiotic	Concentration in medium (μg/ml)	Glucose consumed (μmoles)	Pyruvate produced (μmoles)
None		10·1	18·2
Mitomycin C	200	9·8	17·4
Porfiromycin	200	10·2	17·8
Phleomycin	200	10·0	17·7
Actinomycin D	0·1	9·8	16·9
Pactamycin	200	6·3	6·8
	100	6·6	7·9
	50	8·0	8·6
	25	9·4	9·7

* Trypanosomes (10^8) were suspended in 3·0 ml of calcium-free, phosphate buffer medium (pH 7·6; 0·116 M), containing 30 μmoles glucose and 2% fraction V bovine serum albumin. Incubation in the presence of antibiotics was for 1 hr at 37° in Dubnoff shaking incubator.

of glucose consumed, it would appear from the data in Table 1 that pactamycin was a stronger inhibitor of one or more of the enzymatic reactions leading to the production of pyruvate from hexosephosphate than it was of the phosphorylation of glucose and therefore of its conversion to the biologically active form. The inhibitory effect of pactamycin upon trypanosomal carbohydrate metabolism might therefore account in large measure for its observed depression of nucleic acid and protein metabolism.

Effects on nucleic acid metabolism in vitro

T. equiperdum, as has been found for other trypanosomal species so studied,[22-21] depends primarily upon the anabolism of preformed adenine for total purine nucleotide requirements.[25] It can be seen in Fig. 1 that, in order of potency on a weight basis, actinomycin D, mitomycin C, porfiromycin, and phleomycin inhibited by 50% or more the incorporation of ^{3}H-adenine into trypanosomal RNA *in vitro* at concentrations having no effect on carbohydrate metabolism. Pactamycin, at concentrations causing severe inhibition of carbohydrate metabolism, surprisingly inhibited the incorporation of ^{3}H-adenine into RNA by less than 50%. On a molar basis, the order of potency of the antibiotics at the ID_{50} level for this parameter—considering the approximate molecular weights to be: actinomycin D, 1,284; mitomycin C, 334; porfiromycin, 348; and phleomycin, 1,541—was actinomycin D ($1{\cdot}6 \times 10^{-8}$ M); phleomycin ($1{\cdot}3 \times 10^{-4}$ M); mitomycin C ($2{\cdot}2 \times 10^{-4}$ M); and porfiromycin ($5{\cdot}7 \times 10^{-4}$ M).

Inhibition by these antibiotics of the incorporation of ^{3}H-adenine into RNA was associated with concomitant increases in radioactivity recoverable in the acid-soluble fractions, apparently due in each case to an accumulation of ^{3}H-adenosine triphosphate (Table 2). This pattern suggested that the antibiotics inhibited RNA polymerase.

The effects of these antibiotics upon the incorporation of ^{3}H-adenine into DNA were more selective. At concentrations causing 50% inhibition of the incorporation of this isotope into RNA, actinomycin D had no effect upon its incorporation into DNA, phleomycin was weakly inhibitory, while mitomycin C and porfiromycin were strong inhibitors of this pathway of nucleic acid metabolism (Table 2). It was further found that actinomycin D had no effect upon this parameter even at concentrations causing 80% inhibition of the incorporation of ^{3}H-adenine into RNA.

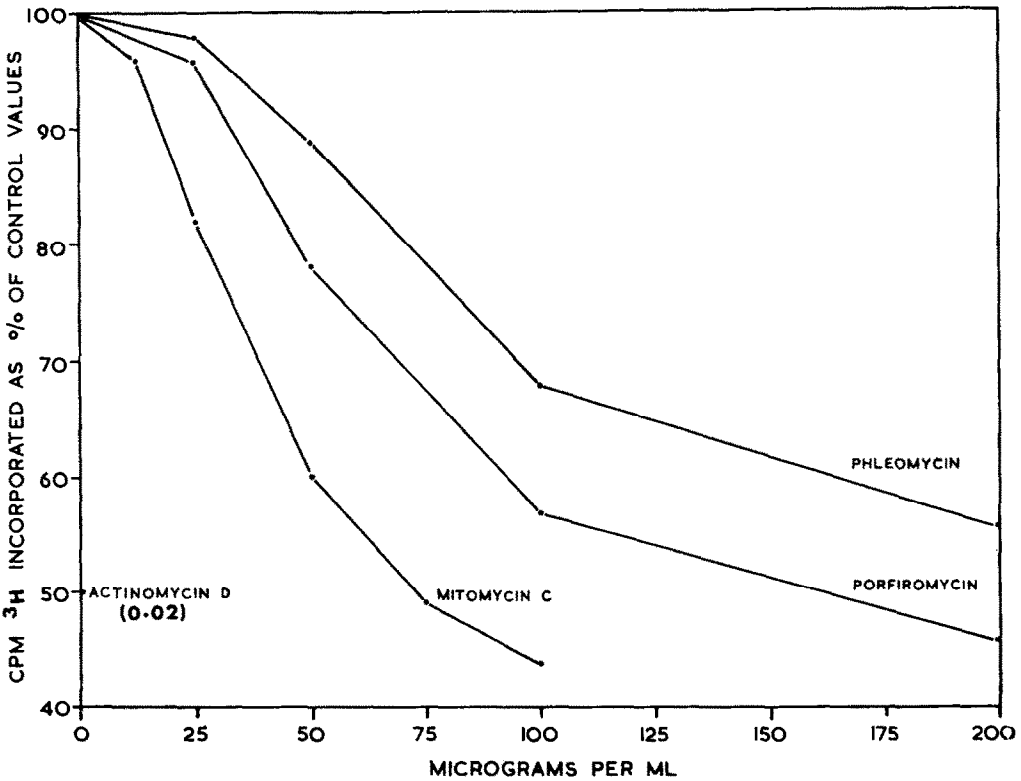

FIG. 1. The effect of actinomycin D, mitomycin C, porfiromycin, and phleomycin upon the ability of *T. equiperdum* to incorporate ^{3}H-adenine into RNA *in vitro*. Suspensions of 10^8trypanosomes in 3·0 ml calcium-free phosphate buffer medium (pH 7·6; 0·116 M) containing 0·01 M glucose and 2% fraction V bovine serum albumin were incubated at 37° in the presence of antibiotics for 75 min and in the presence of ^{3}H-adenine (1 μc in $7{\cdot}7 \times 10^{-4}$ μmole) for 60 min.

T. equiperdum very efficiently can anabolize preformed uracil directly to uridylic acid and thence to polyphosphorylated derivatives that are incorporated into RNA and DNA.[7] It can be seen in Fig. 2 that the ability of actinomycin D, mitomycin C, porfiromycin, and phleomycin to inhibit the incorporation of ^{3}H-uracil into RNA *in vitro* as well as their ID_{50} concentrations for this parameter were quite comparable to that found with regard to effects on the incorporation of ^{3}H-adenine into RNA. In this instance, too pactamycin caused less than 50% inhibition at concentrations causing strong inhibition of carbohydrate metabolism.

Inhibition by these antibiotics of the incorporation of ^{3}H-uracil into RNA was also associated with concomitant increases in radioactivity recoverable in the acid-soluble fractions (Table 3). However, the pattern of distribution of radioactivity in the components of these fractions differed from that found in experiments using ^{3}H-adenine. In the latter case, radioactivity accumulated only in the triphosphonucleotide component. With ^{3}H-uracil as substrate in the presence of the antibiotics, radioactivity accumulated in all components of the acid-soluble fractions. The reason for this observed difference cannot be given at this time. The data, however, still indicated that the inhibitory action of the antibiotics in RNA metabolism was being exerted upon RNA polymerase.

TABLE 2. EFFECT OF ACTINOMYCIN D, MITOMYCIN C, PORFIROMYCIN, AND PHLEOMYCIN ON THE DISTRIBUTION OF RADIOACTIVITY DERIVED FROM ^{3}H-ADENINE IN ACID-SOLUBLE AND NUCLEIC ACID FRACTIONS OF *T. equiperdum in vitro**

Antibiotic	ID_{50}, excluding value in brackets (μg/ml)	Total cpm† in acid-soluble fraction	Distribution of radioactivity (cpm) in acid-soluble fraction				Total cpm in combined nucleic acid fractions	Distribution of radioactivity in nucleic acid fractions	
			A	AMP	ADP	ATP		RNA	DNA
None		146,280	3,140	22,045	48,783	72,312	131,727	128,448	3,279
Actinomycin D	0·02	199,629	3,760	20,458	45,368	130,043	61,939	58,240	3,588
	(0·04)	(230,578)					(42,245)	(38,571)	(3,674)
Mitomycin C	75	187,123	3,139	20,424	53,682	109,878	69,843	67,968	1,875
Porfiromycin	200	176,748	3,174	19,308	46,897	105,369	59,857	58,112	1,745
Phleomycin	200	169,230	3,323	21,447	45,920	98,540	73,432	71,104	2,328

* Experimental conditions as in Fig. 1.
† Average values of triplicate experiments.

TABLE 3. EFFECT OF ACTINOMYCIN D, MITOMYCIN C, PORFIROMYCIN, AND PHLEOMYCIN ON THE DISTRIBUTION OF RADIOACTIVITY DERIVED FROM ^{3}H-URACIL IN ACID-SOLUBLE AND NUCLEIC ACID FRACTIONS OF *T. equiperdum in vitro**

Antibiotic	ID_{50}, excluding value in brackets (μg/ml)	Total cpm† in acid-soluble fraction	Distribution of radioactivity (cpm) in acid-soluble fraction				Total cpm in combined nucleic acid fractions	Distribution of radioactivity in nucleic acid fractions	
			U	UMP	UDP	UTP		RNA	DNA
None		155,545	845	3,160	110,910	40,630	183,812	180,972	2,840
Actinomycin D	0.02	287,227	2,809	6,310	198,576	79,532	93,620	91,500	2,120
	(0·04)	(310,820)					(55,600)	(54,800)	(800)
Mitomycin C	75	270,993	1,789	4,126	184,874	80,204	100,306	90,810	1,496
Porfiromycin	200	262,396	2,108	4,496	180,582	75,210	92,592	91,208	1,384
Phleomycin	200	259,885	2,266	5,084	187,875	64,660	86,230	84,670	1,560

* Experimental conditions as in Fig. 2.
† Average values of triplicate experiment

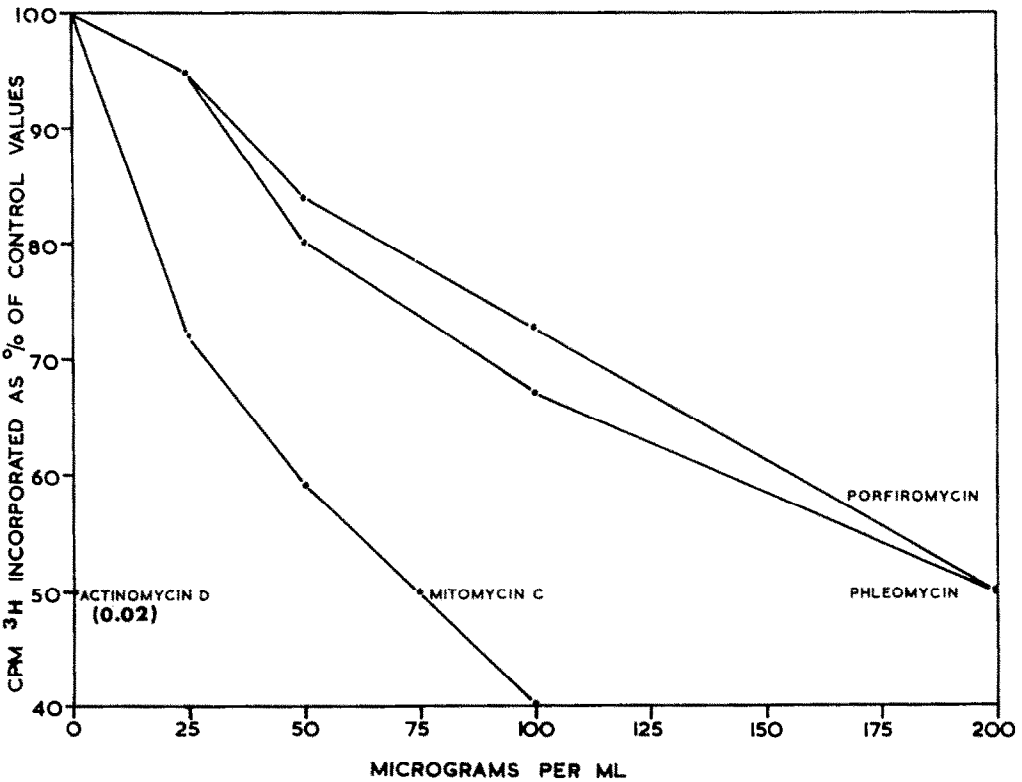

FIG. 2. The effects of actinomycin D, mitomycin C, porfiromycin, and phleomycin upon the ability of *T. equiperdum* to incorporate ^{3}H-uracil into RNA *in vitro*. Suspensions of 10^{8} trypanosomes in 3·0 ml calcium-free phosphate buffer medium (pH 7·6; 0·116 M) containing 0·01 M glucose and 2% fraction V bovine serum albumin were incubated at 37° in the presence of antibiotics for 75 min and in the presence of ^{3}H-uracil (1·0 μc in $3{\cdot}6 \times 10^{-4}$ μmole) for 60 min.

The effects of these antibiotics upon the incorporation of ^{3}H-uracil into DNA (Table 3) differed somewhat from those found in experiments using ^{3}H-adenine. At concentrations causing 50% inhibition of the incorporation of ^{3}H-uracil into RNA, actinomycin D had no effect upon the incorporation of derivatives of this isotope into DNA, but mitomycin C, porfiromycin, and phleomycin were good inhibitors of this pathway of nucleic acid metabolism. It is interesting to note that when the concentration of actinomycin D was increased to a level causing approximately 70% inhibition of the incorporation of ^{3}H-uracil into RNA, this antibiotic then strongly inhibited the incorporation of derivatives of this isotope into DNA (Table 3).

Although *T. equiperdum* can efficiently anabolize preformed uracil *in vitro*, there is evidence to suggest that, *in vivo*, this species depends primarily upon biosynthesis *de novo* of pyrimidines for its total pyrimidine requirements.[3] The presence of this latter metabolic pathway can be demonstrated *in vitro* by the use of orotic acid-^{14}COOH as substrate. *T. equiperdum* can convert orotic acid-^{14}COOH directly to the corresponding monoribonucleotide by its condensation with 1-pyrophosphorylribose-5-phosphate (PRPP), and this nucleotide is then decarboxylated in the presence of orotidylic acid decarboxylase to yield $^{14}CO_2$ and uridylic acid.[7] It can be seen in Fig. 3

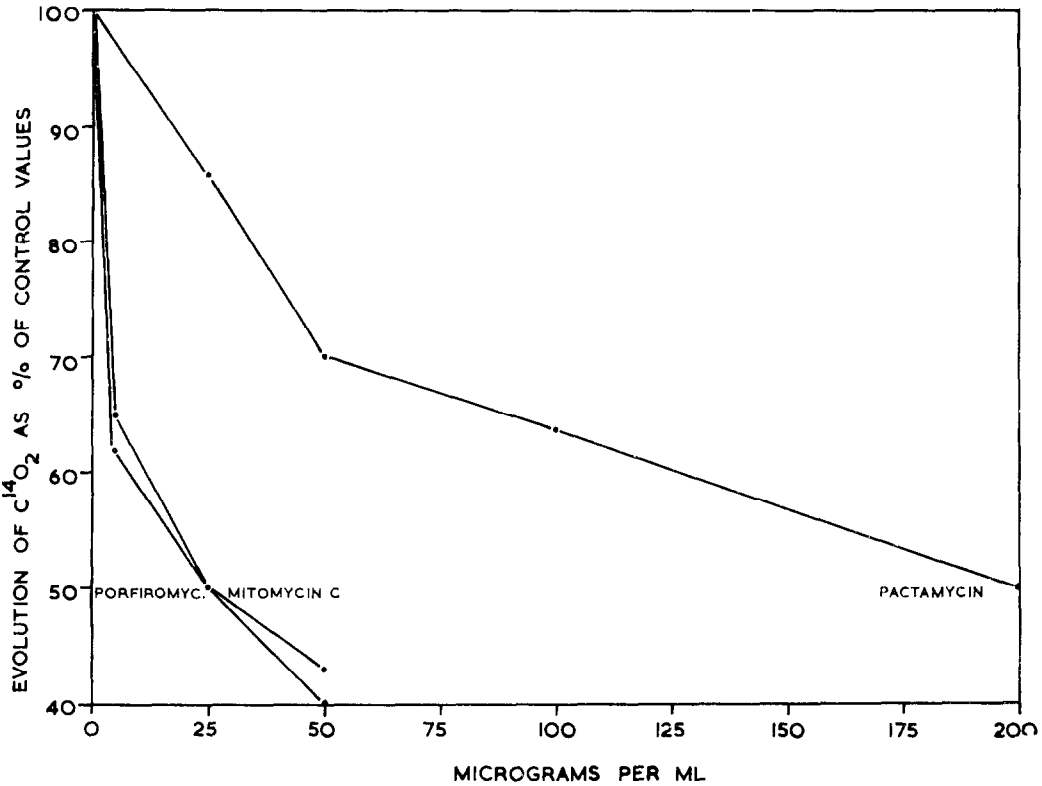

FIG. 3. The effect of mitomycin C, porfiromycin, and pactamycin upon the ability of *T. equiperdum* to convert orotic acid-^{14}COOH to $^{14}CO_2$ and uridylic acid *in vitro*. Suspensions of 10^8 trypanosomes in 3·0 ml calcium-free phosphate buffer medium (pH 7·6; 0·116 M) containing 0·01 M glucose and 2% fraction V bovine serum albumin were incubated at 37° in the presence of antibiotics for 75 min and in the presence of orotic acid-^{14}COOH (0·1 μc in $1{\cdot}2 \times 10^{-2}$ μmole) for 60 min.

that mitomycin C and its methyl congener, porfiromycin, were strong inhibitors of the eventual decarboxylation of orotic acid-^{14}COOH *in vitro* (ID_{50} were $7{\cdot}5 \times 10^{-5}$ M and $7{\cdot}2 \times 10^{-5}$ M respectively), while pactamycin had only marginal activity. Actinomycin D and phleomycin were essentially inactive in this area at concentrations having significant inhibitory potency elsewhere.

A given population of *T. equiperdum* in the bloodstream of the mouse doubles in size every 4·5 to 5·5 hr,[26, 27] and these protozoans retain their ability to synthesize DNA *in vitro* for a limited time after they are removed from the host. It can be seen in Fig. 4 that phleomycin, mitomycin C, and porfiromycin were extremely potent inhibitors of the incorporation of ^{3}H-thymidine into DNA *in vitro* (ID_{50} was $3{\cdot}2 \times 10^{-6}$ M, $7{\cdot}5 \times 10^{-6}$ M, and $1{\cdot}1 \times 10^{-5}$ M respectively). The dose–response curve of pactamycin indicated that, although this antibiotic showed good activity in this area, its inhibition of DNA synthesis was probably secondary to a more pronounced effect of this agent elsewhere, presumably upon carbohydrate metabolism. Actinomycin D had no appreciable effect upon the ability of *T. equiperdum* to incorporate ^{3}H-thymidine into DNA even at concentrations high enough to inhibit the incorporation of derivatives of ^{3}H-uracil into DNA.

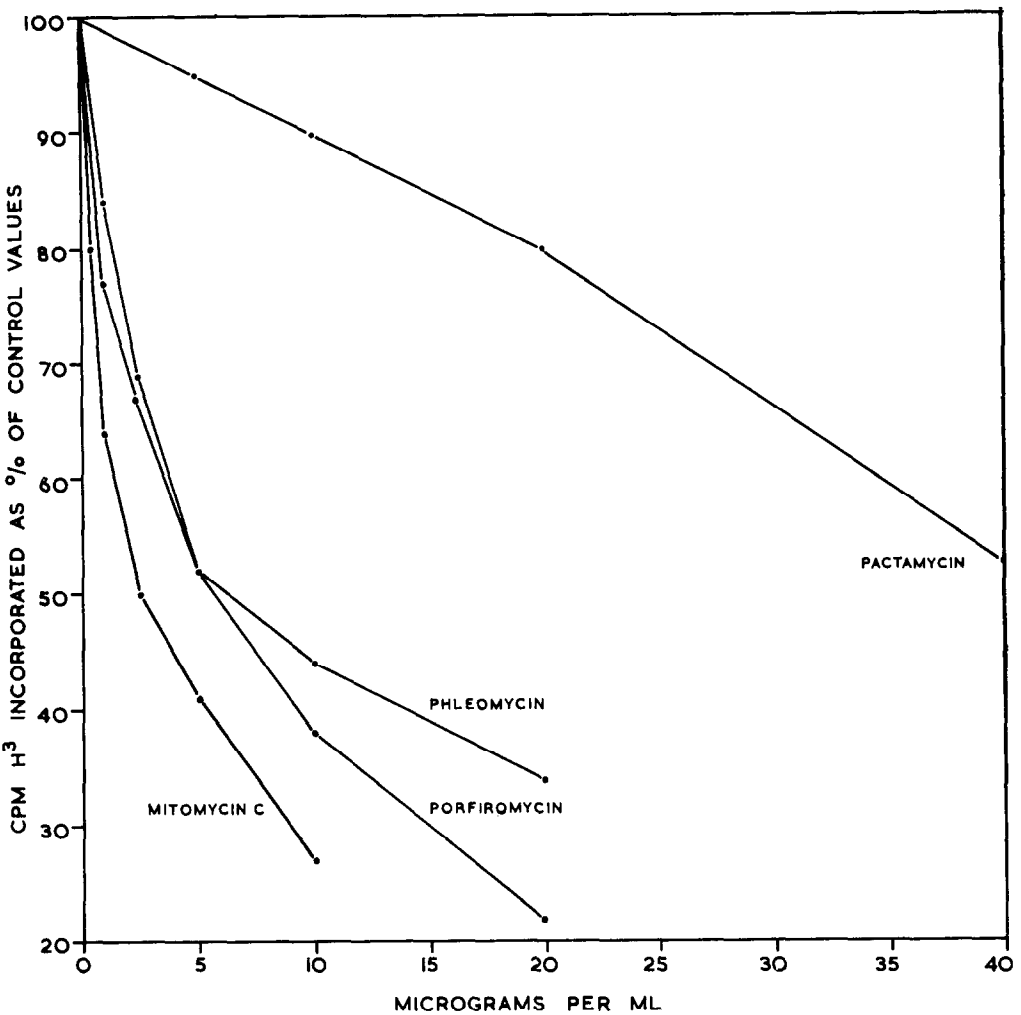

FIG. 4. The effect of mitomycin C, porfiromycin, phleomycin, and pactamycin upon the ability of *T. equiperdum* to incorporate thymidine-CH_3-^3H into DNA *in vitro*. Suspensions of 10^8 trypanosomes in 3·0 ml calcium-free, phosphate buffer medium (pH 7·6; 0·116 M) containing 0·01 M glucose and 2% fraction V bovine serum albumin were incubated at 37° in the presence of antibiotics for 75 min and in the presence of thymidine-CH_3-^3H (1 μc in $1{\cdot}5 \times 10^{-4}$ μmole) for 60 min.

In this instance, inhibition by these antibiotics of the incorporation of ^3H-thymidine into DNA was not associated with concomitant increases in radioactivity recoverable in the acid-soluble fractions nor with detectable changes in the pattern of distribution of radioactivity in the components of these fractions (Table 4). Perhaps the limited extent and time for uptake of this particular isotope under the prevailing experimental conditions made such anticipated changes too small to detect. Since the total uptake of thymidine by drug-treated trypanosomes was less than that of the controls, it is also possible that the antibiotics depressed the rate of passage of thymidine across trypanosomal membranes. Suffice it to say that mitomycin C, porfiromycin, and

TABLE 4. EFFECT OF PHLEOMYCIN, MITOMYCIN C, AND PORFIROMYCIN ON THE DISTRIBUTION OF RADIOACTIVITY DERIVED FROM ^{3}H-THYMIDINE IN THE ACID-SOLUBLE FRACTION AND DNA OF *T. equiperdum in vitro**

Antibiotic	ID_{50} (μg/ml)	Total cpm† in acid-soluble fraction	Distribution of radioactivity (cpm) in acid-soluble fraction				Total cpm in DNA
			TdR	TMP	TDP	TTP	
None		18,829	145	660	6,845	11,179	16,146
Phleomycin	5·0	18,638	240	580	7,210	10,608	8,023
Mitomycin C	2·5	18,536	330	625	5,876	11,705	7,878
Porfiromycin	5·0	18,520	370	507	6,123	11,520	7,963

* Experimental conditions as in Fig. 4.
† Average values of triplicate experiments.

phleomycin did not apparently interfere with the anabolism of thymidine to polyphosphorylated derivatives, but rather with the incorporation of thymidine triphosphate into DNA. The fact that these three antibiotics were so much more potent against the incorporation of thymidine than that of other precursors into DNA makes it less likely that their underlying mode of action was the direct inhibition of DNA polymerase.

Parenthetically, it is of interest that *T. equiperdum* possesses the enzymatic capacity to phosphorylate thymidine, in contrast to its inability to phosphorylate theribo side, uridine.[7] In addition, this species was found unable to anabolize thymine, again in contrast to its efficient anabolism of uracil.

Effects on protein metabolism in vitro

The rapid turnover of trypanosomal populations in the blood stream of the host is obviously associated with considerable protein synthesis. It has been assumed that the source of amino acids for trypanosomal protein synthesis is the host plasma rather than the products of trypanosomal glucose metabolism,[28] and Aronson* has recently found that *T. equiperdum* can incorporate at least 16 different amino acids into protein *in vitro*, phenylalanine being taken up to the greatest extent. It can be seen in Table 5 that, except for pactamycin, none of the antibiotics affected the incorporation of phenylalanine-U-^{14}C into protein at concentrations having significant inhibitory potency elsewhere. On the other hand, pactamycin markedly inhibited protein synthesis and, although this action may in all likelihood be secondary to the effect of pactamycin upon carbohydrate metabolism, the possibility cannot be ruled out that pactamycin can interfere specifically with some phase of protein synthesis.

DISCUSSION

Reference was made earlier to several protozoan species that have been proven useful for the detection and investigation of antitumor agents. One of these is the African trypanosome, *T. equiperdum*, the sensitivity of which to various tumor-inhibitory antibiotics is the subject of this report. The accumulating evidence that this and other species of trypanosomes[3, 7, 8, 29, 30] can be used for mechanistic studies of a

* Personal communication.

TABLE 5. EFFECT OF TUMOR-INHIBITORY ANTIBIOTICS UPON THE ABILITY OF *T. equiperdum* TO INCORPORATE PHENYLALANINE-U-^{14}C INTO PROTEIN *in vitro**

Antibiotic	Concentration in medium (μg/ml)	Radioactivity recovered (cpm)	
		Acid-soluble	Protein
None		600	39,807
Actinomycin D	0·1	620	40,916
Mitomycin C	100	740	38,170
Porfiromycin	200	800	48,700
Phleomycin	200	900	36,000
Pactamycin	1·0	310	3,735
	0·75	480	4,699
	0·5	590	9,904
	0·25	610	30,627
	0·1	610	47,277

* Suspensions of 10^8 trypanosomes in 3·0 ml calcium-free phosphate buffer medium (pH 7·6; 0·116 M) containing 0·01 M glucose and 2% fraction V bovine serum albumin were incubated at 37° in the presence of antibiotics for 75 min and in the presence of phenylalanine-U-^{14}C (1·0 μc in 2·7 $\times$ 10^{-3} μmole) for 60 min.

variety of compounds that interfere with carbohydrate, protein, and nucleic acid metabolism seems particularly timely, since it has been recently demonstrated that several trypanocidal drugs with little or no antitumor activity per se can potentiate the action of such antitumor agents as azaserine, terephthalanalides, and methylglyoxal-bis-guanylhydrazone.[31–33] It is obvious that elucidation of the modes of action of these and other trypanocidal drugs may contribute directly to a better understanding of their efficacy in cancer chemotherapy.

The data presented herein indicate that the inhibitory action of phleomycin, mitomycin C, porfiromycin, and actinomycin D in *T. equiperdum* was directed against various pathways of nucleic acid metabolism. The author is unaware of reports concerning the mode of action of pactamycin in other systems; therefore, the finding that pactamycin was a potent inhibitor of trypanosomal carbohydrate metabolism and possibly of protein synthesis awaits confirmation by others.

Phleomycin, isolated from *Streptomyces verticillus*,[34] is a water-soluble, copper-containing peptide complex, effective against a variety of bacteria[35] and experimental neoplasms.[36–38] Studies of its mode of action in *Escherichia coli* and HeLa cells *in vitro*[39] indicated that phleomycin selectively inhibited DNA synthesis, as evidenced by its inhibition of the incorporation of ^{3}H-thymidine into this cellular component. Studies using the Kornberg synthetic system indicated that phleomycin preferentially inhibited DNA polymerase, probably by binding DNA primer.[40] At higher concentrations, phleomycin also inhibited RNA polymerase. At minimal growth-inhibitory concentrations for *E. coli*, phleomycin did not interfere significantly with carbohydrate metabolism, protein synthesis, or the incorporation of ^{32}P into RNA.[39]

Similarly, phleomycin did not, under prevailing experimental conditions, interfere with the carbohydrate metabolism and protein synthetic capacity of *T. equiperdum*. The predominant feature of its action upon trypanosomal nucleic acid metabolism *in vitro* was inhibition of the incorporation of ^{3}H-thymidine into DNA, the ID_{50} of phleomycin for this parameter being, on a molar basis, approximately one fortieth of that causing comparable inhibition of the incorporation of derivatives of ^{3}H-uracil and ^{3}H-adenine into either DNA or RNA. While such action certainly reflected

interference by phleomycin with DNA replication, it remains unclear why the incorporation of ^{3}H-thymidine was more susceptible to the action of phleomycin than was the incorporation of other DNA precursors, if the underlying action of phleomycin is to bind DNA primer or template and thus to inhibit DNA polymerase. It is tempting to speculate that in *T. equiperdum* phleomycin might bind predominantly to adenine residues of the DNA template, thus preventing DNA polymerase-mediated base pairing of adenine to thymine. There is of course no evidence at present to support this assumption.

The ability of phleomycin to inhibit the incorporation of labeled precursors into RNA, and the concomitant changes it caused in the pattern of distribution of radioactivity in the various components of the acid-soluble fraction, indicated that this antibiotic also interfered with RNA polymerase.[41] Although the marked sensitivity of *T. equiperdum* to actinomycin D indicates that its RNA polymerase system is predominantly, if not exclusively, DNA-directed,[42, 43] it is not possible to state at this time whether phleomycin inhibited RNA polymerase directly or whether its ability to inhibit RNA synthesis in this species was a consequence of its action against DNA synthesis.

Mitomycin C, isolated from *Streptomyces caespitosus*,[44] and its methyl congener, porfiromycin, isolated from *S. ardus*,[45] are pyrroloindole benzoquinone derivatives,[46] effective against a broad spectrum of bacteria,[47, 48] and a number of experimental neoplasms.[49–51] Studies of their mode of action in bacterial[52–54] and mammalian systems[55–57] indicated that both compounds, qualitatively similar in all respects, acted selectively upon DNA, the primary action being either to promote the breakdown or depolymerization of pre-existing DNA or to inhibit the synthesis or utilization of DNA precursors. More recent evidence suggested that the mitosane antibiotics, after reduction, alkylate complementary DNA strands, thus reducing the availability of effective DNA primer.[58–60]

The action of mitomycin C and porfiromycin in *T. equiperdum* was qualitatively similar in all respects. At concentrations causing no interference with carbohydrate metabolism or protein synthesis, both antibiotics inhibited the incorporation of various labeled precursors into RNA and DNA. The predominant action of the mitosane antibiotics, as was also found to be the case for phleomycin, was inhibition of the incorporation of ^{3}H-thymidine into DNA, the ID_{50} for this parameter also being, on a molar basis, approximately one fortieth of that causing comparable inhibition of the incorporation of derivatives of ^{3}H-uracil and ^{3}H-adenine into either RNA or DNA. Thus in certain respects, the inhibitory effects of the mitosane antibiotics were found to be quite similar to those of phleomycin, as would be expected if the underlying mechanism in both instances was interference with the efficacy or availability of the DNA template. In fact, there is other evidence to suggest that the mode of action of phleomycin is qualitatively similar to that of the mitosane group, but quantitatively milder.[39]

In contrast to the very weak action of phleomycin in this area, both mitomycin C and porfiromycin inhibited the evolution of $^{14}CO_2$ derived from orotic acid-^{14}COOH, whether by direct interference with orotidylic acid decarboxylase or by some other mechanism remaining unsettled. Presumably, the phosphorylribosidation of orotic acid via PRPP was not blocked, since the mitosane antibiotics did not affect the ability of *T. equiperdum* to convert uracil directly to its corresponding monoribonucleotide.

Actinomycin D, first isolated from *Streptomyces chrysomallus*,[61] is a phenoxazinpeptide derivative[62] effective against Gram-positive but not Gram-negative bacteria[63] and possessing selective antitumor activity in animals[64] and man.[65, 66] Studies of its mode of action have indicated that actinomycin D combines with DNA[67, 68] and, as a consequence, inhibits DNA-directed RNA polymerase.[42, 43, 69] DNA polymerase can be inhibited by actinomycin D, but at much higher concentrations of the antibiotic than are required to inhibit RNA polymerase.[70]

At concentrations causing no interference with the carbohydrate metabolism and protein synthetic capacity of *T. equiperdum*, actinomycin D strongly inhibited the ability of this species to synthesize RNA. The distribution of radioactivity in the various components of the acid-soluble fractions of trypanosomes exposed to actinomycin D strongly suggested that this antibiotic was exerting its action upon RNA polymerase.

The effect of actinomycin D upon trypanosomal DNA metabolism deserves comment. The incorporation of tritiated thymidine (as well as adenine) was totally unaffected by even very high concentrations of the antibiotic. Apparently, in *T. equiperdum*, the anabolism of thymidine to the triphosphate form and the intrinsic activity of the DNA polymerase system were quite resistant to the action of actinomycin D. Yet it was found that high concentrations of this antibiotic inhibited the incorporation of radioactivity derived from tritiated uracil into DNA. This would suggest that actinomycin D inhibited either (1) synthesis *de novo* of deoxycytidine triphosphate or (2) synthesis *de novo* of thymidine monophosphate or both. If this newly revealed facet of actinomycin's mode of action be confirmed, it could be due either to direct inhibition by the antibiotic of specific pertinent enzyme systems or be a reflection of the failure of new enzyme formation as a consequence of the primary inhibition of RNA polymerase and RNA synthesis.

The marked sensitivity *in vitro* of one or more metabolic pathways in *T. equiperdum* to actinomycin D, mitomycin C, porfiromycin, phleomycin, and pactamycin indicated that these antibiotics might be trypanocidal *in vivo*. Results obtained so far have revealed wide disparities in their efficacies in the treatment of experimental trypanosomiasis. However, the curative potentials of several of these antibiotics, particularly actinomycin D, phleomycin, and porfiromycin, seem to be sufficiently dramatic to warrant a more detailed report in the near future.

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