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STUDIES ON THE ANTIMICROBIAL ACTION OF TEREPHTHALANILIDE- AND RELATED DRUGS*

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Abstract—The drug 2-chloro-4′,4″*bis*(2-imidazolin-2-yl)-terephthalanilide dihydrochloride (NSC 38280) and other polycationic growth inhibitors of *E. coli* have been examined with particular reference to their inhibition of protein synthesis. Of nine polycationic drugs studied, NSC 38280 and three congeners, as well as spermine and stilbamidine, were found to be inhibitors of protein synthesis on the basis of their ability to uncouple the regulatory control of protein synthesis upon RNA synthesis.

At a high level, NSC 38280 inhibits the incorporation of isoleucine-U^{14}C into polypeptide in a cell-free system derived from *E. coli*. In this system the soluble fraction and, to a lesser extent, the ribosomes are sensitive to the drug. NSC 38280 does not cause the release of nascent protein from ribosomes *in vitro*. In intact cells, NSC 38280 slightly lowers the charging of amino acids upon transfer RNA, in contrast to a slight increase encountered on treatment with chloramphenicol.

Cellular uptake of NSC 38280 is heterogeneous, being dependent in part on an active transport sensitive to 2,4-dinitrophenol administration, and in part on an adsorption which increases upon lowering of temperature. A fraction of the uptake is resistant to washing of intact cells and is also resistant to dialysis of crushed cell preparations. Upon cellular fractionation, the affinity of drug appears to be poor on the ribosomes, but high on a component released from the ribosomes upon washing.

Although NSC 38280 inhibits growth and incorporation into protein in the intact cell immediately, it becomes bactericidal only after 20 min. The inhibitory effects are reversed to differing extents by polyamines, particularly by spermine. No consistent correlation was found between the degree of reversal of protein synthesis and reversal of lethality. It is concluded that inhibition of protein synthesis is the first event in the action of NSC 38280, but cell death ensues upon the development of a more general, pervasive physiological action of the drug.

The growth-inhibiting effects of several polycatronic drugs on the trypanosomid flagellate *Crithidia fasciculata* are reported.

OF THE SEVERAL studies that have been made thus far of the antimicrobial action of the terephthalanilide drugs,[1] two particularly salient facts emerge from the behavior of NSC 38280.† First, a large, inexclusive number of nutritional precursors of proteins

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† The following abbreviations are used: ATP, adenosine triphosphate; 2,4-DNP, 2,4-dinitrophenol; CAL, chloramphenicol, MeGAG, methylglyoxal bis(guanylhydrazone)HCl; MeGMS, methyglyoxal bis (N^4 methylthiosemicarbazone); NSC 38280, 2-chloro-4′,4″*bis*(2-imidazolin-2-yl)-terephthalanilide; NSC 50469, 2-amino-4′,4″*bis*(2-imidazolin-2-yl)terephthalanilide; NSC 53212 4′,4″*bis* (2-imidazolin-2-yl)-isophthalanilide; NSC 53306, 4′4″*bis* (2-imidazolin-2-ylamino)-terephthalanilide; 53312, 4′4″-*bis*(*N*′methylamidino)-terephthalanilide; NSC 57155, *N, N*″-*bis*[*p* (*N*′-methylamidino)phenyl]-terephthalamidine; *t*-RNA, transfer RNA. All basic drugs were used as the hydrochlorides.

and nucleic acids can partly, but never fully, alleviate growth inhibition.[2, 3] Second, the primary biosynthetic effect in the intact cell is to inhibit protein synthesis, which uncouples RNA synthesis from the normal control of protein synthesis, and allows some DNA synthesis to complete the round initiated by previous cellular growth.[4] The inhibition of protein synthesis by NSC 38280 differs from that with CAL in that the uncoupled production of RNA is not as extensive, and no immature ribosomes are formed. Indeed NSC 38280 prevents part of the appearance of excess RNA with CAL.[4] It would therefore appear that although inhibition of protein synthesis is a major physiological effect, the drug may in fact be more multifarious in its sites of inhibition or in the effects of its prime action.

The results to be presented are part of an investigation to determine more specifically the indicated site of inhibition of protein synthesis and to look for indications of other possible causes of growth inhibition.

The study is extended to include a comparison of polycationic drugs that either bear structural similarities to NSC 38280 or have been implicated to have pharmacological similarities in differing biological systems. Some of these drugs have been used as trypanocidal agents, and it was of interest to determine the toxicity of the collection of drugs used in this study on the trypanosomid, *Crithidia fasciculata.*

METHODS

The routine organism used in these studies is the *E. coli* strain 113–3 of Davis, auxotrophic for either methionine or vitamin B_{12}. Other organisms that are specified are wild type *E. coli* B, the phenylalanine-requiring *E. coli* ATCC 9723f, and the tryptophan-requiring *E. coli* ATCC 9662. Media and conditions of growth have been described.[4]

C. fasciculata was cultivated on a complex, defined medium[5] in stagnant layers or with very slow shaking at room temperature.

Procedures for the orcinol determination of RNA or the Folin-biuret determination of protein, and for harvesting, preparation, and radioactive counting of cellular products and cellular fractions have been previously described.[4, 6] Procedures of ultra-centrifugal analysis have been previously described.[4] Amino acids of the intracellular pool were estimated by centrifuging the cells at room temperature, resuspending, and finally extracting the cell pellet with 10% cold trichloroacetic acid at 0°C, extracting the acid with ether. The remainder was made alkaline with NaOH, aerated while boiling to remove NH_3, and assayed for amino acids colorimetrically with ninhydrin,[7] with alanine as a standard.

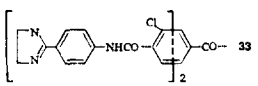
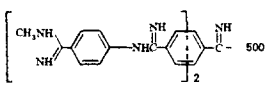
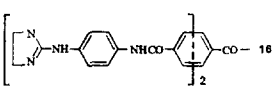
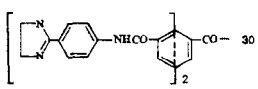
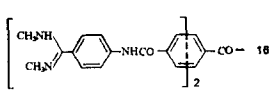
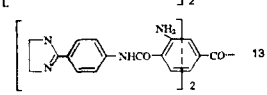
NSC 38280 was assayed by the colorimetric estimation of primary aromatic amine released from it on acid hydrolysis.[8] Cell-free protein synthesis was followed with a preparation of washed, dialyzed ribosomes and dialyzed soluble fraction, as described by Matthaei and Nirenberg.[9]

RESULTS

Comparative effects of the terephthalanilide drugs on E. coli. It has been suggested, on the basis of drug synergism and tumor cross resistance, that the many active compounds that have been obtained by structural modification of the original terephthalanilide drugs may comprise in fact several pharmacological classes with differing

physiological action.[10,11] It is therefore not unexpected to find divergence among the drugs listed in Table 1 and Table 2 in their ability to selectively inhibit protein synthesis in *E. coli*. This ability is judged from the induction of abnormally high proportions of cellular RNA, which is characteristic of inhibitors of protein synthesis. Of the congeners of NSC 38280 listed in Table 1, the drugs NSC 57155, NSC 53306, and NSC 53212 are effective in raising RNA/protein ratios, and the drugs NSC 53312 and

TABLE 1. INHIBITION OF *E. Coli* BY PHTHALANILIDE AND RELATED DRUGS AND THEIR EFFECT ON THE CELLULAR LEVEL OF RNA*

NSC#	Formula	Conc μg/ml	% Inhibition of growth	RNA/ Protein
No Drug				Init. 0·42 Final. 0·33
38280		33	95	0·78
57155		500	100	0·64
53306		16	78	0·63
53212		30	66	0·57
53312		16	33	0·46
50469		13	89	0·46

* A culture of *E. Coli* 113–3 was grown in minimal medium with methionine to a cell turbidity of 60 Klett–Summerson units and treated for 90 min, with drugs most of which were graded in 3- to 3·3-fold doses. Growth was followed turbidimetrically, and cultures showing appreciable or extensive growth inhibitions at the most minimal drug levels were selected for RNA/protein determinations.

NSC 50469 are ineffective. The drugs were additionally examined twice or more in separate experiments with slight modifications of exposure. NSC 57155 and NSC 53306 at high growth-inhibitory concentrations consistently caused increases in RNA/protein ratios of 20–60 per cent, whereas NSC 53312 and NSC 50469 were not significantly effective. With NSC 53212, however, the ability to elevate selectively the proportions of cellular RNA, illustrated in Table 1, was not consistent and sometimes not distinguishable. Likewise, elevation of the RNA proportions with very high concentrations of spermine, as shown in Table 2, was sometimes difficult to reproduce. In Table 2 stilbamidine is shown to be an effective selective inhibitor of protein synthesis only at partially inhibitory concentrations. Elevating the drug level evidently

increases side effects to the point where the inhibition of protein synthesis is no greater than that of RNA synthesis. It has not been possible to show any selective inhibition of protein synthesis with propamidine at any concentration (Table 2 and unpublished experiments). With CAL or NSC 38280, any concentration giving a growth inhibition in excess of 20–30 per cent will elevate the proportion of cellular RNA. To sum up, the polycations probably have a multitude of physiological effects, inhibition of protein synthesis predominating with certain compounds sometimes only at limited

TABLE 2. INHIBITION OF *E. coli* BY POLYCATIONIC DRUGS AND THEIR EFFECT ON THE CELLULAR LEVEL OF RNA*

Drug	Conc. (μg/ml)	Inhibition of growth (%)	RNA/protein
None	Initial sampling		0·52
	Final		0·55
CAL	2	76	1·10
	1	32	0·62
	0·5	23	0·54
	0·25	10	0·47
	0·13	5 (stim.)	0·43
NSC 38280	30	82	0·86
	7·5	58	0·58
	3·8	24	0·65
	1·8	2 (stim.)	0·59
Stilbamidine	167		0·58
	50	64	0·77
	17	47	0·87
	5		0·71
	1·7		0·59
Propamidine	167	86	0·55
	50	64	0·54
	17		0·47
Spermine	1200	66	0·79

* Experimental conditions are the same as in Table 1.

ranges of concentration. Thus the selective inhibition of protein synthesis by NSC 38280 is preserved upon conversion of the amide to amidino groups accompanied by opening of the imidazoline ring to give an amidine derivative (NSC 57155). However, ring opening alone abolishes selective activity (NSC 53312). Inter-position of a pair of secondary amino groups within the molecule retains activity (NSC 53306), but the addition of an amino substituent of the terephthalic acid nucleus abolishes it (NSC 50469). Rearrangement to the isophthalanilide form (NSC 53212) reduces but does not abolish selective inhibition of protein synthesis.

Effect of NSC 38280 *on cell-free protein synthesis.* To demonstrate a direct effect of NSC 38280 on polypeptide synthesis in an isolated system,[9] a fairly high concentration of the drug is required, but not a level that precipitates protein or ribosomes (Expt. 1, Table 3). The drug has little effect when preincubated with messenger RNA (Expt. 1, Table 3) upon which the system is highly dependent (Expt. 2, Table 3). However, at a concentration that initially amounts to 100 μg/ml before dilution for assay, the drug can severely impair either the washed ribosomes or the supernatant fraction containing *t*-RNA and the enzymes for polypeptide assembly. Drug addition at the level of 29 μg/ml at the start of incorporation results in a more limited inhibition, at best halving the activity (Expt. 1 and 2, Table 3). Although the extent of inhibition

varied from preparation to preparation, the ribosomes were always less susceptible than the supernatant fraction to preincubation with the drug. Seemingly, either the *t*-RNA or the accessory enzymes of polypeptide assembly are most susceptible to the drug.

Effects of polycationic drugs on ribosomal profiles. NSC 38280, streptomycin, MeGAG, and spermine were examined for their ability to alter the ultracentrifugal

TABLE 3. EFFECT OF NSC 38280 ON POLYPEPTIDE SYNTHESIS BY CELL-FREE PREPARATIONS FROM *E. coli* B*

Expt.	Treatment	Isoleucine-U-^{14}C incorporation	
		(cpm/ sample)	(% inhib.)
1	Complete system: components preincubated 10 min at 37°		
	No drug present	168	
	10 μg NSC 38280 added upon mixing components	136	19
	10 μg NSC 38280, preincubated:		
	with template RNA, in 0·05 ml	79	53
	with supernatant protein (S 100), in 0·1 ml	12	93
	with ribosomes, in 0·1 ml	22	87
2	Complete	241, 256	
	+ 10 μg NSC 38280 added after 15 sec	150, 141	41
	Minus template RNA	11	96

* The reaction mixture contained the following: supernatant protein (S 100 fraction) containing 950 μg RNA in 0·1 ml; washed ribosomes containing 528 μg RNA in 0·1 ml; template RNA containing 55 μg RNA in 0·05 ml; 0·1 μg of each of the amino acids of a complete mixture; 0·05 μc of isoleucine-U-^{14}C and all other ingredients as specified by Matthaei and Nirenberg[9] in a final volume of 0·35 ml. The reaction was started by the addition of amino acids and supplements, incubating in a 37° water bath for 30 min. Template RNA was prepared from a phenol extraction of broken *E. coli* cells that had been infected for 10 min with T4 phage at a 10-fold multiplicity of infection. Samples were counted on a low background gas flow counter.

profile of isolated ribosomes or of unfractionated broken cell preparations. In the presence of 0·01 M Mg acetate and at concentrations that do not precipitate ribosomes, the only significant effect that could be produced by these agents was ribosomal dimerization to produce an 88s peak, obtainable only with spermine. Dimerization was not further modified by the addition of NSC 38280, nor did NSC 38280 by itself alter the dissociated ribosomal profile, mostly of 30s and 50s components, in 0·003 M Mg acetate. Thus, antiribosomal effects do not appear to be signaled by any striking gross effect on the ultracentrifugal patterns, except for precipitation.

Effect of NSC 38280 *on the charging of amino acids on* t-RNA. With $^{35}SO_4$, a precursor of methionine and cystine (Expts. 1 and 2, Table 4), and tryptophan-3-^{14}C (Expt. 3, Table 4) as representative amino acid sources, the fate of their transitional attachment to *t*-RNA before their incorporation into protein was followed during normal and drug-inhibited growth. The cells were first equilibrated for 5 min with a steady state supply of amino acid, treated with drug, and the *t*-RNA was harvested by extraction in hot 10 per cent NaCl and recovery from 67 per cent ethanol.[12] CAL produces a consistent 50 per cent increase in the accumulated cellular level of amino acyl *t*-RNA. This increase would be expected, since the biosynthetic step subsequent to *t*-RNA loading is the site sensitive to the drug. In contrast, NSC 38280 depresses this

level, either in absolute terms, or at least in the sense that the increase encountered in CAL treatment is not found. If this were the main site of growth inhibition, the ribosomal demand for charged *t*-RNA might be expected to have stripped the *t*-RNA more completely of its amino acid label. It is alternately possible that the drug binds and inactivates both charged and uncharged *t*-RNA with similar affinities, the level of persisting amino acid thus being immaterial to the extent of inhibition.

TABLE 4. EFFECT OF NSC 38280 AND CHLORAMPHENICOL ON CHARGING OF AMINO ACIDS ON TRANSFER RNA IN *E. coli*

Expt	*E coli strain*	Source, concentration and duration of		Incorporation in RNA (cpm/mg RNA)
		Amino acid	Drug	
1	113–3 with vitamin B_{12} replacing methionine	$Na_2{}^{35}SO_4$, 0·25 μc in 10 μg ml, 0–25 min	None	1430, 1690
			NSC 38280, 30 μg/ml, 5–25 min	780
			CAL, 30 μg/ml, 5–25 min	2480
2	As above	$Na_2{}^{35}SO_4$, 0·028 μc in 6 μg/ml, 0–40 min	None	710, 700
			NSC 38280, 20 μg/ml, 15–40 min	669, 560
			CAL, 20 μg/ml, 15–40 min	934, 920
		As above, with 30 μg/ml each of methionine and cystine, 0–40 min		14
3	*E coli*/trypt	0·067 μc DL tryptophan 3 ^{14}C in 5 μg ml, 0–15 min	None	215
			NSC 38280, 20 μg/ml, 5–15 min	225
			CAL, 20 μg/ml, 5–15 min	335

TABLE 5. DISTRIBUTION OF PULSE-LABELED PHENYLALANINE-U-^{14}C IN *E. coli* AND THE EFFECT OF NSC 38280 TREATMENT *in vitro**

Subcellular fraction	Specific activity		Total activity	
	Treated (cpm/mg protein)	Control	Treated (cpm in protein)	Control
Supernatant protein	302	277	225	224
Membranes and wall ghosts	718	548	1099	804
Ribosomes	1650	1110	316	556
Total			1640	1584

* Procedure: phenylalanine-requiring *E. coli* was starved of phenylalanine for 20 min and pulsed for 5 sec with 5 μc of L-phenylalanine-U-^{14}C (0·044 μc in 0·02 μg/ml), iced, washed, broken by freeze-thawing with lysozyme and DNAase in the medium of Matthaei and Nirenberg,[9] diluted to 4·6 mg cell protein/ml supplemented with 1·3 mg Na_2ATP/ml and incubated for 5 min with 30 μg NSC 38280/ml and then fractionated, maintaining the drug at the same concentration in the washings. Growth, pulsing and the 5-min drug incubation were at 37°. All other manipulations were at 0°.

Effect of NSC 38280 *on the attachment of nascent protein to ribosomes.* Next examined was the possibility of altering the normal cellular distribution of nascent protein. As indicated in Table 5, the distribution of pulse-labeled protein in cell preparations that have been broken after brief amino acid labeling strikingly illustrates the identity of the nascent protein by its preferential attachment to the ribosomes. Specific activity of the ribosomal protein is four times that of the supernatant fraction. More prolonged labeling would distribute the isotope essentially equally in all fractions.[6] The initial exposure to NSC 38280 for 5 min at 37° is adequate to allow the drug to exert its effect *in vitro* while some polypeptide synthesis takes place. The only effect of such treatment is to coacervate part of the ribosomal fraction, which then is recovered with the membranes (Table 5). Therefore, under conditions where some inhibition of cell-free synthesis can be demonstrated (Table 3), the drug does not disrupt the integrity of the association between nascent protein and the ribosome.

Cellular uptake and subcellular distribution of NSC 38280. The uptake of NSC 38280 by *E. coli* appears to be a heterogeneous process, as indicated in Expt. 1, Table 6. That

TABLE 6. UPTAKE AND SUBCELLULAR LOCALIZATION OF NSC 38280 IN *E. coli*

NSC 38280 dosage	Expt. 1 (15 μg/ml, 1 hr)	Expt. 2 (10 μg/ml, 1 hr)
(Cell preparation*)	(Cellular uptake in recovered protein) (μg drug/mg protein)	
Cells incubated at 0°, unwashed	151	
at 37° with 10^{-3} M 2,4-DNP, unwashed	36	
at 37°, unwashed†	62	
washed, broken, dialyzed‡	6·0	
undialyzed	7·5	
walls		15·6
walls and coarse membrane		11·7
membranes, combined		10·8
membranes, coarse	12·9	
membranes, fine	12·3	
ribosomes, unwashed	6·9	
washed	3·0	2·7
washings from above	45	19·8
supernatant protein	8·4	8·4

* Cultures of *E. coli* 113–3 were exposed to drug at a cell turbidity of 100 Klett-Summerson units, centrifuged at the temperature of incubation and swabbed free of liquid, washed, broken in the French pressure cell and centrifugally fractionated, all at 0° and in 0·01 M Tris (hydroxymethylamino) methane succinate, pH 7·4 + 0·01 M Mg acetate. Procedural details have been previously described.[3, 5]

† In terms of cell uptake, the level is equal to 453 μg drug/ml of cell pellet.

‡ Dialysis was done in cellophane casing for 16 hr at 7° in 0·15 M saline with stirring.

part of the uptake is energy-dependent is evident from its inhibition by about 40 per cent on phosphorylative uncoupling with 2,4-DNP. However, uptake is increased over 2-fold if incubation is carried out at 0°, indicating, on the other hand, that much of the uptake would also consist of passive adsorption on the bacterial surface. A single washing removes much of the drug that is associated with the cells. The residuum, however, must be tightly bound to the macromolecular constituents of the cell, since the drug level is only slightly affected by overnight dialysis (Expt. 1, Table 6). On subfractionation (Expt. 1 and 2, Table 6), attachment of the drug is found to be

heightened in the walls and in the membranes. The smallest drug level is associated with the ribosomes. On a total weight basis, these values would actually be lower than indicated, since the ribosomes contain more RNA than protein. After washing, most of this uptake passes into the supernatant fraction where its level seems strikingly elevated. The main components that would be most likely to occur in the supernatant would be some of the uncentrifuged ribosomal components, probably the 30s fraction, and *t*-RNA, which is strongly associated initially with the centrifuged ribosomes and is then easily dissociated on washing.[13]

Comparison of the effects of NSC 38280 *on protein biosynthesis and on cell viability.* The relevance of the antagonism of protein biosynthesis by NSC 38280 to its lethal action was investigated by determining how closely the two events are linked. There is no lag in the arrestment of cellular turbidity by the drug.[4] Correspondingly, in Fig. 1

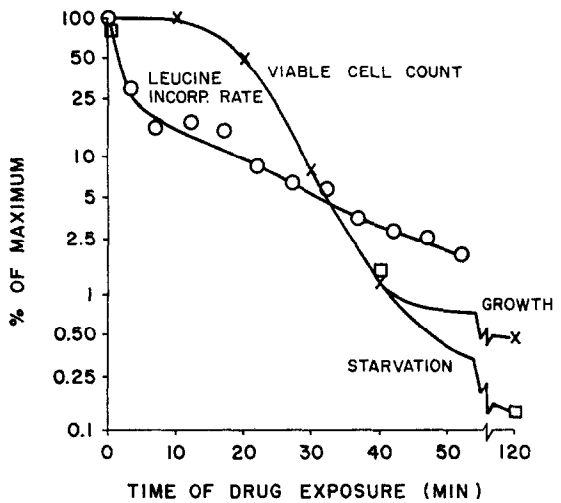


FIG. 1. The course of action of NSC 38280 on *E. coli:* lethality of the drug at 30 μg/ml and its inhibition of leucine incorporation into protein. Cells in complete medium or in medium deficient in NH_3 and methionine were plated on nutrient agar for counting (100 per cent = $4{\cdot}1 \times 10^8$ colony-forming cells/ml). Incorporation of leucine was followed by plating 1-ml samples of the growing culture for 4 min with 5 μg of leucine-1-^{14}C containing 10^4C cpm, followed by inactivating plating and washing the cells on cellulose filters with 10 per cent trichloroacetic acid (100 per cent activity = 1012cpm). The values of leucine incorporation are drawn at the midpoint of isotope exposure.

the onset of inhibition of protein incorporation is seen to be instantaneous. Its most pronounced development, occurring within 20 min, seems to be overcome by diluting and plating the cells. Subsequent drug lethality is not accompanied by a comparable further drop in protein synthesis that could account for the irreversible consequences. Although turbidity increases slightly during cell death with NSC 38280, growth is not needed, for starving cells are not resistant to the drug (Fig. 1).

The antibacterial effects of NSC 38280 can be alleviated with increasing effectiveness by the polyamines putrescine, spermidine, and spermine. As seen in Fig. 2, they differ markedly in preserving viability. Their restoration of leucine incorporation, in contrast, is ultimately fairly uniform. Spermine can also reverse as well as prevent

drug toxicity. When it follows the drug by 2 min, antagonism is actually slightly improved, reflecting perhaps some converse drug alleviation of the slight variable toxicity of spermine itself that is encountered at this high level. If spermine addition is delayed as much as 40 min, about two-thirds of the maximal growth restoration is evident by the time a further 80 min have elapsed.

Miscellaneous observations: membrane integrity, lipid synthesis, and respiration of E. coli *during treatment with* NSC 38280. Significant loss of membrane semipermeability with NSC 38280 was not demonstrable. A 40-min treatment of cultures with 20 μg/ml of drug only slightly lowers the intracellular amino acids from their normal level of 0·80 μmole/mg cell protein to 0·63; however, the value does remain unchanged at 0·79 after comparable treatment with 10μg CAL/ml.

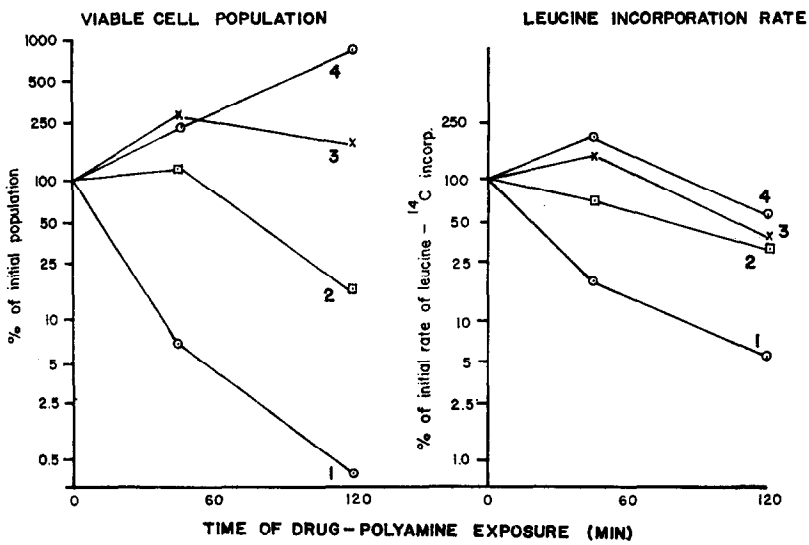


FIG. 2. Antagonism by polycations of the action of NSC 38280 on cell viability and on leucine incorporation. NSC 38280 was present at 15 μg/ml. Other conditions and methods are as described in Fig. 1. Curve 1 drug alone. Drug was added simultaneously with 2 mg/ml levels of the hydrochlorides of putrescine (curve 2), spermidine (curve 3), and spermine (curve 4). Initial population = 3·1 × 10^8 cells/ml; initial incorporation = 1257 cpm.

Although NSC 38280 can strikingly inhibit lipid synthesis in the L 1210 ascites tumor,[14, 15] it has no such effect in *E. coli*. Treatment of a culture with 30 μg/ml of NSC 38280 for 40 min did not affect the incorporation of acetate-1-^{14}C into the cellular lipids extractable with a 1:1 mixture of chloroform:methanol.

O_2 uptake of glucose cultures is unaffected by treatment for 2 hr with either NSC 38280 or NSC 53212 at 30 μg/ml. A similar treatment with NSC 38380 was also found not to affect the endogenous respiration of washed cells. Therefore, the phthalanilides do not exert any impairment of respiration. Also, since they do not stimulate respiration, they probably do not uncouple phosphorylation.

Effects on growth of C. fasciculata. *C. fasciculata* would appear to be a useful test organism for evaluating toxocity *in vitro* of some polycationic drugs. It is a trypanosomid related to the pathogens susceptible to stilbamidine and propamidine treatments,

and it is cultivatable on a defined medium.[5] Table 7 details the growth inhibitions of some drugs tested. They do not increase the RNA proportions of the cell abnormally, but no conclusions may be drawn from such results, since it is not known whether this organism is indeed capable of attaining high levels of uncoupled RNA synthesis. The organism was found not to be susceptible to treatment with CAL or puromycin,

TABLE 7. EFFECT OF NSC 38280 AND RELATED DRUGS ON GROWTH AND RNA LEVELS IN *C. fasciculata**

Expt.	Drug	Conc. for 40–60 per cent growth inhibition (μg/ml)	RNA/protein
1	None		
	Initial growth		0·27, 0·26
	Final		0·16, 0·15
	Stilbamidine	150	0·16
	Propamidine	15	0·16
	NSC 38280	15	0·17
2	MeGMS	3	
	NSC 53312	10	
	NSC 53306	>30, <100	
	MeGAG	400	
	NSC 53212	70	
	Spermine	1000	
3	NSC 57142	>100	
	NSC 57155	>100	
	Synthalin	>100	
	NSC 50469	<100	

* Procedure: a 5 per cent inoculum of *C. fasciclata* was grown for 2 days in the presence of drug. Growth was estimated by cell protein determination or by direct cell counts. Normal growth increase varied from 12-fold to 50-fold in different experiments.

which would have established a useful comparison of drug effects. The effects of both NSC 38280 and NSC 53212, which are moderately inhibitory, are partly prevented by a mixture of nutrient broth and yeast extract; those of the other drugs are not.

DISCUSSION AND CONCLUSIONS

The anticipated inhibition of protein synthesis by the phthalanilide drugs was borne out in a study of cell-free polypeptide synthesis (Table 3), with comparatively high drug levels. It is not the ribosome itself but rather the supernatant fraction that appears most susceptible to NSC 38280. It is evident from Table 4 that NSC 38280 does not exert its action by detaching nascent protein from the ribosome *in vitro*, as would puromycin. The site of protein biosynthesis whose inactivation would be most likely explained by the effects encountered experimentally would be *t*-RNA. An explanation has been offered for the limited decrease of amino acid loading on *t*-RNA, as shown in Table 4, on the basis of siimlar affinities of the drug for both charged and uncharged forms of *t*-RNA. The phthalanilide drugs may then derepress RNA synthesis by altering the regulatory response of the cell to *t*-RNA. It has been proposed that *t*-RNA regulates RNA synthesis by the extent of its charging with amino acid.[16] It is possible that drug binding to *t*-RNA would also alter the recognition of *t*-RNA by a regulatory site, and would thus produce the same effect as that exerted by CAL at a subsequent site

on the ribosome. Since these effects on *t*-RNA recognition might not be identical, the overproduction of RNA in the presence of phthalanides might not be efficient, and could, under some circumstances, even fail (Table 1).

The interpretation of *t*-RNA binding in the action of NSC 38280 would also be in accord with the drug's most selective concentration in ribosomal washings. It has been noted that the ribosomes of *E. coli* may carry much of the cellular *t*-RNA with them during their first centrifugation,[13] and lose this fraction on washing.

That the inhibition of protein synthesis by NSC 38280 represents only part of a spectrum of physiological events is borne out by the seeming lack of close association between the inhibition of protein incorporation and the bactericidal effect of the drug. For example, a slight addition to the already precipitous fall in protein incorporation shown in Fig. 1 precedes a wave of lethality. At the same time, as shown in Fig. 2. putrescine fails to preserve cellular viability, although it preserves much of the original incorporative capacity of the cell, not far less efficiently than spermidine or spermine. This lack of correlation may indeed apply to the limitations among the phthalanilide drugs in uncoupling RNA synthesis (Table 1). This could reflect the predominance of other sites of drug action rather than differences in the particulars of inhibition of protein synthesis.

Of considerable relevance in the interpretation of these findings are the high binding affinities of terephthalanilide drugs and their congeners for nucleic acids.[17–19] Excellent correlations have been established between drug toxicity for *Lactobacillus casei* and drug activity in displacing complexes of DNA with dye[18] and in elevating melting points of duplex DNA strands.[19] Although the studies have been primarily concerned with drug–DNA complexes, physiological interactions of the drugs with RNA appear as likely,[17, 19] and indeed the difference spectra produced by a drug complex with DNA and that with RNA were strikingly similar, indicating that the drug interaction is of a general and nonselective nature. It may then be postulated that one form of RNA, possibly *t*-RNA, is the initial target of drug binding, but that subsequently the drug affinities permeate among all nucleic acids. If sequential binding with DNA inhibits further DNA and RNA synthesis, this could account for the limitations that have been shown in the uncoupled production of RNA and of DNA in *E. coli* during growth inhibition by NSC 38280.[4]

A select number of polybasic drugs are active growth inhibitors of *C. fasciculata* (Table 7). It is of interest that drugs with basic structures similar, to, or derived from, those of NSC 38280 and NSC 53312, which are among the most active against *C. fasciculata*, are highly active against experimental murine infections with *Trypanosoma brucei*, and to a lesser extent, with *T. congolense*.[20]

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