Biochemical Pharmacology, 1962, Vol. 11, pp. 453–466. Pergamon Press Ltd., Printed in Great Britain.

THE FIVE-MEMBERED DISULPHIDE RING SYSTEM—II

CYTOSTATIC EFFECTS

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(*Received* 19 *October* 1961; *accepted* 6 *November* 1961)

Abstract—1:2-Dithiolane derivatives have been shown to exert a cytostatic effect on strain L mouse fibroblasts *in vitro*. A similar effect could usually not be demonstrated on Ehrlich's ascites tumour cells *in vivo*, although these tumour cells were more sensitive to the antirespiratory effect of the 1:2-dithiolane derivatives than the fibroblasts. Evidence is presented that the lack of cytostatic activity *in vivo* is due to rapid elimination.

THE resemblance pointed out by Schotte and Nygård[1] between 1:2-dithiolane derivatives and diselenides in their polarographic behaviour suggests that the five-membered disulphide ring system may have a pronounced effect on cell metabolism and cell growth. This led to a study of the cytostatic effect of a number of 1:2-dithiolane derivatives on Earle's strain L mouse fibroblasts *in vitro* and on Ehrlich's ascites tumour cells *in vivo*. Furthermore, the effect on respiration of these two types of cells and on glycolysis in Ehrlich's ascites tumour cells was investigated. The results reported in the present paper show that some 1:2-dithiolane derivatives have a cytostatic and an anti-respiratory effect *in vitro*. However, under the conditions of the present experiments these compounds with one exception showed no significant cytostatic effect *in vivo*. Some reasons for this are pointed out.

MATERIALS AND METHODS

The compounds are listed in Table 1, which also shows the chemical formulae and the LD_{50} doses in mice. Apart from α-lipoic acid, which was obtained from Chemiewerk, Homburg, Frankfurt/Main, all the compounds were synthesized at the University Institute of Chemistry at Uppsala by Schotte. The LD_{50} doses on single intraperitoneal injections into mice were determined at the research laboratories of Pharmacia, Uppsala.

Earle's strain L mouse fibroblasts (929) were grown in a modified type of Carrel flasks in a fluid medium containing 30 per cent horse serum, 10 per cent chick embryo extract and 60 per cent Tyrode's solution. The medium was renewed daily.

The Ehrlich ascites tumour used in the present investigation was a hypotetraploid subline carried in inbred mice of the St/Eh strain.

The effect on cellular respiration was studied by means of the Cartesian diver technique.[2, 3] For these experiments non-hemorrhagic Ehrlich's ascites tumours and Earle's strain L mouse fibroblasts grown in suspension cultures under permanent magnetic stirring were used. The cells were suspended in Ringer–Lock's solution. The gas phase was air.

For the study of aerobic glycolysis Ehrlich's ascites tumour cells were suspended in a Krebs–Ringer phosphate solution with 5 mM glucose and with air as the gas phase. Glucose and lactic acid concentrations were determined enzymically as described by Huggett and Nixon,[4] and by Horn and Bruns.[5] Determinations were made before the experiment and after 3 hr of incubation.

RESULTS

The growth inhibiting effect on Earle's strain L fibroblasts are shown in Figs. 1–10. At from 2 to 3 days' interval, from two to four cultures from each experimental group

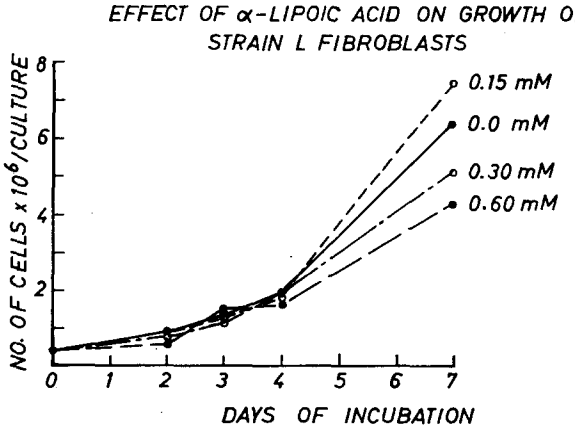


FIG. 1. Effect of α-lipoic acid on growth of strain L fibroblasts.

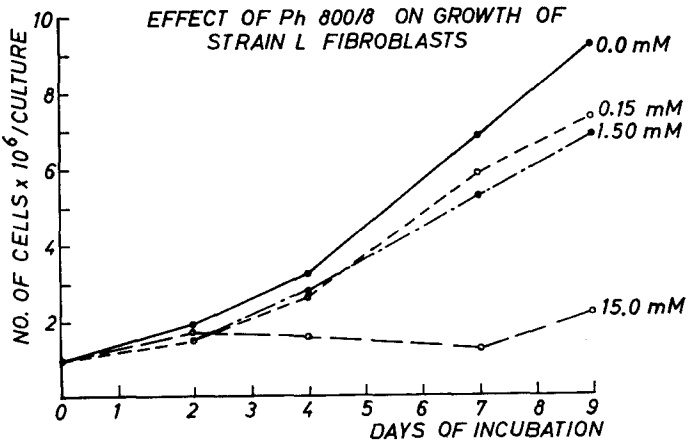


FIG. 2. Effect of Ph 800/8 on growth of strain L fibroblasts.

were trypsinized at 37 °C for 10 min with a 0·5 per cent trypsin solution. The cell concentration in the resulting suspensions was determined in a Bürker–Türk haemocytometer, and the total cell number per flask was calculated. The curves shown in the figures have been drawn on basis of average values thus obtained.

The various compounds were added to the cultures at different concentrations chosen on basis of the LD_{50} values shown in Table 1. However, since all compounds were tested at a concentration of 0·15 mM, their cytostatic effects at equimolar concentration could be compared as shown in Table 2.

TABLE 1

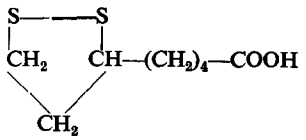
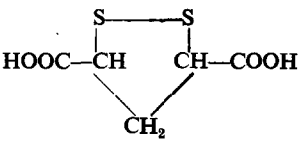
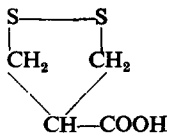
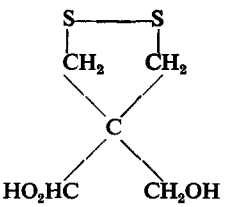
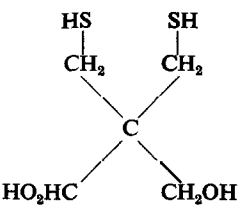
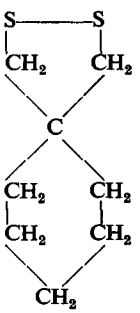
Compound	Code no. of compound	Formula	LD_{50} in mice (single i.p. injection)
α-Lipoic acid	—		—
1:2-Dithiolane-3:5-di-carbonic acid	Ph 800/8		2–4 g/kg
1:2-Dithiolane-4-carbonic acid	Ph 800/21		27 mg/kg
4:4-Bis-hydroxymethyl-1:2-dithiolane	Ph 800/7		120 mg/kg
Reduced form	Ph 800/57		124 mg/kg
2:3-Dithio-4:5-spirodecane	Ph 800/2		136 mg/kg

TABLE 1 (*continued*)

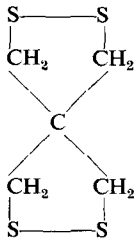
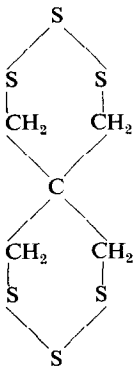
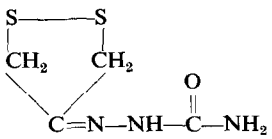
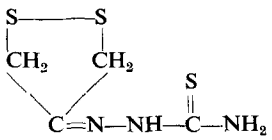
Compound	Code no. of compound	Formula	LD_{50} in mice (single i.p. injection)
2:3:7:8-Tetrathio-(4:4)-spirononane	Ph 800/20		25 mg/kg
2:3:4:8:9:10-Hexathio-(5:5)-spiroundecane	Ph 800/19		> 4000 mg/kg
1:2-Dithiolanon-4-semicarbazone	Ph 800/1		34 mg/kg
1:2-Dithiolanon-4-thiosemicarbazone	Ph 800/18		65 mg/kg

TABLE 2. CYTOSTATIC EFFECTS OF 1:2-DITHIOLANE DERIVATIVES AT 0·15 mM CONCENTRATION ON L-STRAIN FIBROBLASTS GROWN FOR ONE WEEK *in vitro*

Compound	Relative cell number (per cent of control)
α-lipoic acid	117
Ph 800/8	87
Ph 800/21	47
Ph 800/7	90
Ph 800/57	72
Ph 800/2	23
Ph 800/20	79
Ph 800/19	73
Ph 800/1	8
Ph 800/18	13

From the curves and the table it is seen that α-lipoic acid at 0·15 mM concentration was without any cytostatic effect, while Ph 800/8, Ph 800/57, Ph 800/7, Ph 800/20 and Ph 800/19 had a very moderate effect. The two last mentioned compounds were almost insoluble in the medium. Ph 800/21 had a more pronounced effect. In spite of

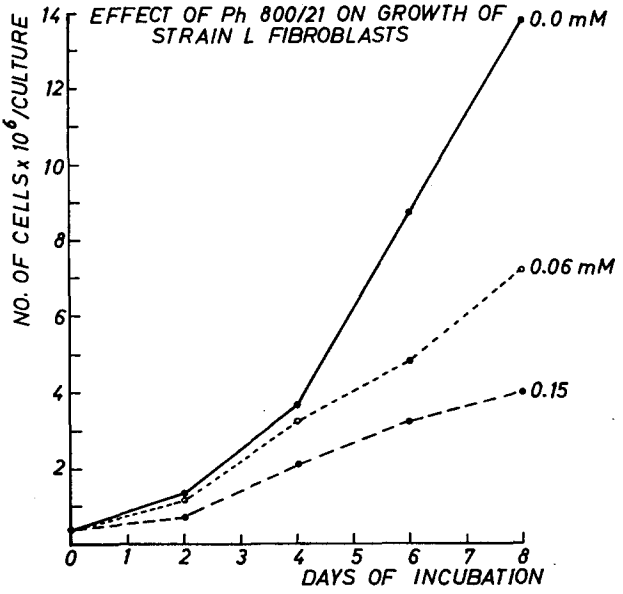


FIG. 3. Effect of Ph 800/21 on growth of strain L fibroblasts.

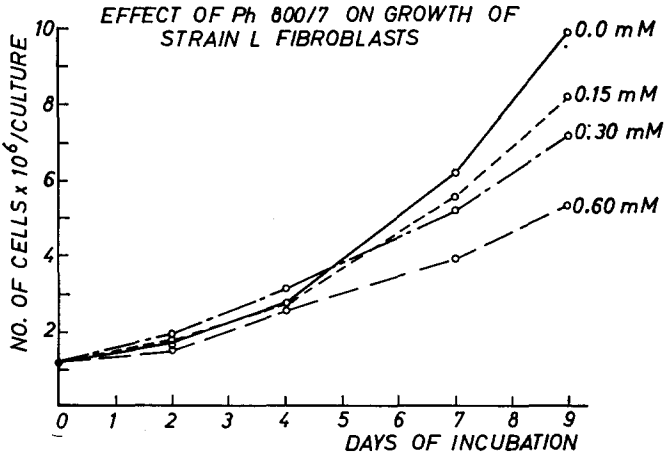


FIG. 4. Effect of Ph 800/7 on growth of strain L fibroblasts.

its low solubility Ph 800/2 had a considerable effect, and an even greater effect was shown by Ph 800/18 and Ph 800/1, which suppressed cell growth completely.

If the cytostatic effects of the various compounds are compared not on basis of equimolarity, but rather on basis of the LD_{50} doses as shown in Table 3, similar

results were obtained with the exception that Ph 800/8 at this concentration showed a considerable effect. Also the effect of Ph 800/57 was quite pronounced. Thus, the strain L mouse fibroblasts seem to be sensitive particularly to Ph 800/1, Ph 800/18, Ph 800/2, Ph 800/8, Ph 800/57 and Ph 800/21.

TABLE 3. CYTOSTATIC EFFECTS OF 1:2-DITHIOLANE DERIVATIVES AT LD_{50} DOSES ON L-STRAIN FIBROBLASTS GROWN FOR ONE WEEK *in vitro*

Compound	LD_{50} (m-moles/kg)	Conc. *in vitro* (mM)	Relative cell number (per cent of control)
Ph 800/8	10–20	15	18
Ph 800/21	0·18	0·15	47
Ph 800/7	0·72	0·6	64
Ph 800/57	0·74	0·6	31
Ph 800/2	0·78	0·6	25
Ph 800/20	0·13	0·15	79
Ph 800/1	0·19	0·15	8
Ph 800/18	0·34	0·15	9

TABLE 4. CYTOSTATIC EFFECTS OF 1:2-DITHIOLANE DERIVATIVES ON EHRLICH'S ASCITES TUMOUR CELLS *in vivo*

Treatment		Median number of cells × 10^6 per mouse*		
Compound	Daily dose (m-moles/kg)	Controls	Treated animals	Per cent of control
α-lipoic acid	0·15	17·6 ± 10·0	13·2 ± 9·6	75
Ph 800/8	0·15	30·9 ± 19·4	33·9 ± 20·8	110
	1·50	29·6 ± 15·8	3·9 ± 3·5	13
Ph 800/21	0·06	45·3 ± 13·5	49·8 ± 18·5	110
Ph 800/7	0·15	58·1 ± 11·0	46·4 ± 9·3	80
Ph 800/57	0·15	63·8 ± 17·6	66·3 ± 18·2	104
Ph 800/2	0·15	42·9 ± 12·4	55·2 ± 27·7	129
Ph 800/20	0·15	25·4 ± 11·3	27·2 ± 23·9	107
Ph 800/19	0·15	30·9 ± 19·4	34·6 ± 19·7	112
Ph 800/1	0·03	25·4 ± 11·3	19·4 ± 17·8	76
Ph 800/18	0·15	41·0 ± 13·1	40·0 ± 18·6	98

* ± range x confidence factor at the 95 per cent level.[6]

In order to test the cytostatic effects on Ehrlich's ascites tumour *in vivo*, one million cells were transplanted i.p. into St/Eh mice weighing 20 g. Each experimental group comprised five females and five males. Treatment was started on the day of transplantation and continued for 5 days. Each day the mice received one intraperitoneal injection with the compound under investigation dissolved or suspended in 0·2 ml Tyrode's solution. On the fifth day all the animals were sacrificed. The ascites fluid was removed as completely as possible, and afterwards the peritoneal cavity was

washed with 5 ml Tyrode's solution. The ascites fluid and the washing fluid were pooled and after a cell count in Bürker–Türk's haemocytometer the total cell number per mouse was calculated. The results were subjected to statistical analysis, using Dean and Dixon's method for small number of observations.[6] Table 4 shows the doses used, the median cell number, and the confidence limits at the 95 per cent level.

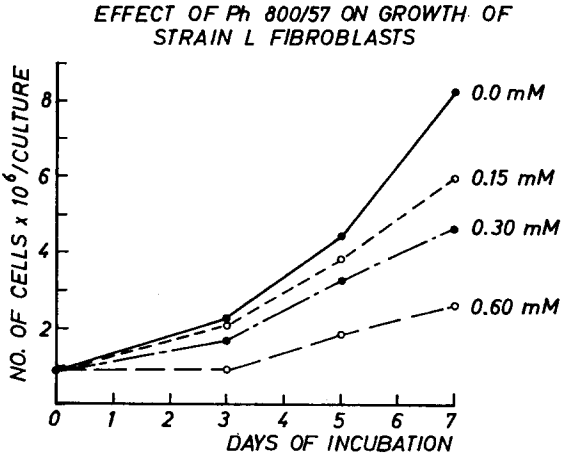


FIG. 5. Effect of Ph 800/57 on growth of strain L fibroblasts.

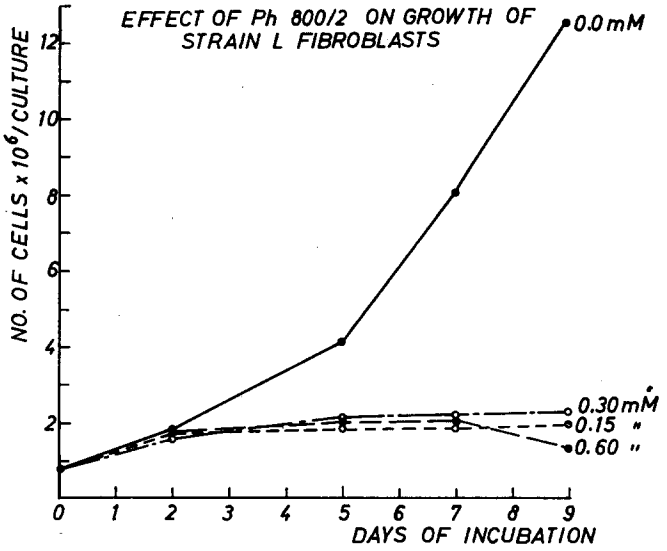


FIG. 6. Effect of Ph 800/2 on growth of strain L fibroblasts.

As it is seen only Ph 800/8 at a dose of 1·5 m-moles per kg had a significant growth-inhibiting effect.

These results suggest that the Ehrlich ascites tumour cells are resistant to 1:2-dithiolane derivatives. However, studies of their effect on cellular respiration do not support this idea.

Table 5 shows the results of two experiments with each compound. It appears that at 0·15 mM concentration only Ph 800/1 and possibly Ph 800/2 had a pronounced effect on the respiration of Earle's strain L fibroblasts. All the other compounds showed only a moderate inhibitory effect or no effect. The respiratory metabolism of

TABLE 5. EFFECT OF 1:2-DITHIOLANE DERIVATIVES AT 0·15 mM CONCENTRATION ON CELLULAR RESPIRATION

Compound	Rate of respiration (per cent of control)	
	Strain L fibroblasts	Ehrlich's tumour cells
α-lipoic acid	82	79
	98	85
Ph 800/8	99	60
	99	69
Ph 800/21	74	84
	86	85
Ph 800/7	72	56
	74	62
Ph 800/57	76	63
	98	63
Ph 800/2	63	60
	76	69
Ph 800/20	73	99
	90	100
Ph 800/19	100	91
	102	98
Ph 800/1	53	33
	66	34
Ph 800/18	79	43
	82	55

TABLE 6. EFFECT OF 1:2-DITHIOLANE DERIVATIVES AT 0·15 mM CONCENTRATION ON AEROBIC GLYCOLYSIS IN EHRLICH'S ASCITES TUMOUR CELLS

Compound	Rate of glycolysis (per cent of control)	
	Glucose consumption	Lactic acid production
α-lipoic acid	103	97
Ph 800/8	139	119
Ph 800/21	140	104
Ph 800/7	90	77
	105	93
Ph 800/57	122	101
Ph 800/2	148	128
Ph 800/20	113	111
	120	109
Ph 800/19	109	103
	78	93
Ph 800/1	125	156
	126	136
Ph 800/18	167	140
	136	108

the Ehrlich cells showed a greater sensitivity than the L cells to the inhibitory effect of Ph 800/8, Ph 800/7, Ph 800/57, Ph 800/1 and Ph 800/18.

If the cytostatic effects of 1:2-dithiolane derivatives are due to an interference with energy metabolism the higher rate of glycolysis in Ehrlich's ascites tumour cells may account for the apparent resistance of these cells. In Table 6 the results of some

experiments are shown, which demonstrate that at 0·15 mM the 1:2-dithiolane derivatives were without any inhibitory effect on aerobic glycolysis. The results rather indicate a slight stimulatory effect on glucose consumption and lactic acid production by some of the derivatives.

Rapid detoxification or removal from the peritoneal cavity of the 1:2-dithiolane derivatives might also explain the discrepancy between the effects on growth *in vivo* and respiration *in vitro* of the Ehrlich ascites tumour cells.

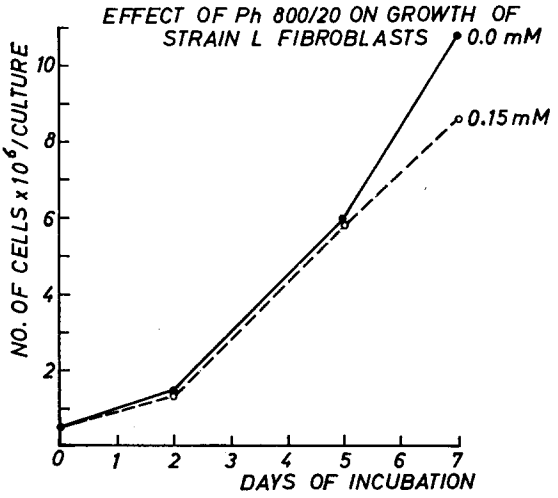


FIG. 7. Effect of Ph 800/20 on growth of strain L fibroblasts.

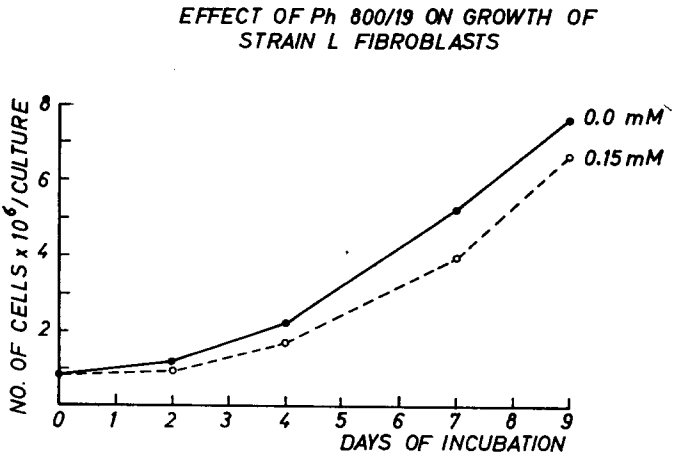


FIG. 8. Effect of Ph 800/19 on growth of strain L fibroblasts.

In order to elucidate this possibility the growth inhibitory effect on the L strain cells was used as a method for the demonstration of the two most active 1:2-dithiolane derivatives, Ph 800/1 and Ph 800/18 in the ascites fluid and in the tumour cells 1–2 hr after injection.

Two experiments were carried out. In the first one thirty mice carrying the Ehrlich ascites tumour received 0·2 m-moles i.p. of Ph 800/1 per kg body weight. Ten mice died within 15 min after the injection. Of the twenty survivors ten were killed after 1 hr and ten after 2 hr. The ascites fluid was removed immediately, and after centrifugation the supernatant was stored at 3 °C. At the same time a 0·2 mM solution of Ph 800/1 in Tyrode's solution was made up and stored under similar conditions. Ascites fluid from non-treated animals served as control material.

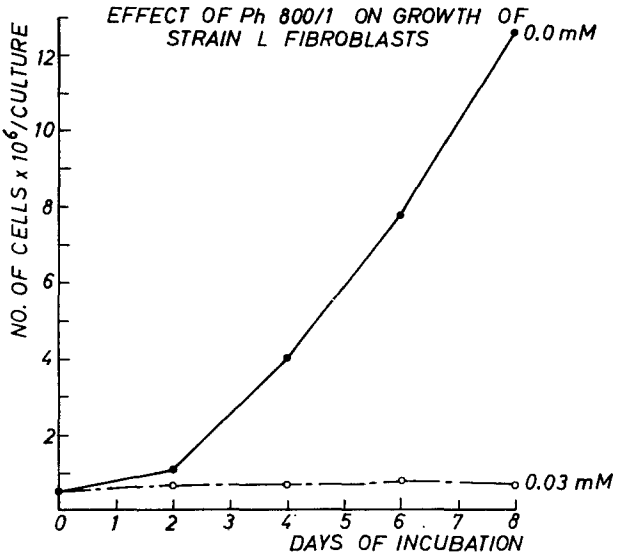


FIG. 9. Effect of Ph 800/1 on growth of strain L fibroblasts.

In order to test the stored Tyrode solution and the ascites fluids for Ph 800/1 activity, strain L cultures containing an initial number of $1{\cdot}3 \times 10^6$ cells were fed the standard medium with the material to be tested added at a concentration of 10 per cent. This should give a final Ph 800/1 concentration of 0·02 mM in the cultures receiving the Tyrode's solution, unless the compound was broken down during storage. The control cultures received 10 per cent ascites from non-treated animals.

The media were renewed daily. After 4 days the cultures were trypsinized and the number of cells counted. The figures shown in Table 7 are average values of from three to four cultures. It is seen that the Ph 800/1 activity in the Tyrode's solution was retained during storage, while the ascites fluid from treated animals showed very little if any activity. This indicates that the compound in less than 1 hr is either bound almost completely to the cells of the tumour, or inactivated, or removed from the peritoneal cavity.

In a similar experiment with Ph 800/18 the ascites fluid was pooled from fifteen mice which 1 hr earlier had received 0·5 m-moles per kg body weight by the intraperitoneal route. After centrifugation the supernatant ascites was removed and stored at 3 °C. The tumour cells were resuspended in a small volume of distilled water and disintegrated by repeated freezing and thawing. After addition of Tyrode's solution up to the original volume the homogenate was shaken vigorously with glass beads for

1 hr. Microscopical control showed that no intact cells were left. Finally the homogenate was centrifuged and the supernatant cell extract was removed and stored. Pooled ascites tumours from non-treated animals were prepared in a similar way and stored together with a 0·5 mM solution of Ph 800/18 in Tyrode.

The test was carried out with groups of from three to six cultures which were fed daily with a medium containing 90 per cent standard medium and 10 per cent of the

TABLE 7. ELIMINATION OF PH 800/1 FROM EHRLICH'S ASCITES TUMOUR IN 1–2 HR
(Cytostatic effect of ascites on L-strain fibroblasts)

Supplement to culture medium	No. of cells × 10^6 per flask*	
	Initial	After 4 days
10% ascites from non-treated mice	1·3	5·0
10% ascites withdrawn from treated mice 1 hr after injection of 0·2 m-moles Ph 800/1 per kg body weight	1·3	4·1
10% ascites withdrawn from treated mice 2 hr after injection of 0·2 m-moles Ph 800/1 per kg body weight	1·3	4·7
10% Tyrode's sol. with 0·2 mM Ph 800/1	1·3	1·0

* Average of three to four cultures.

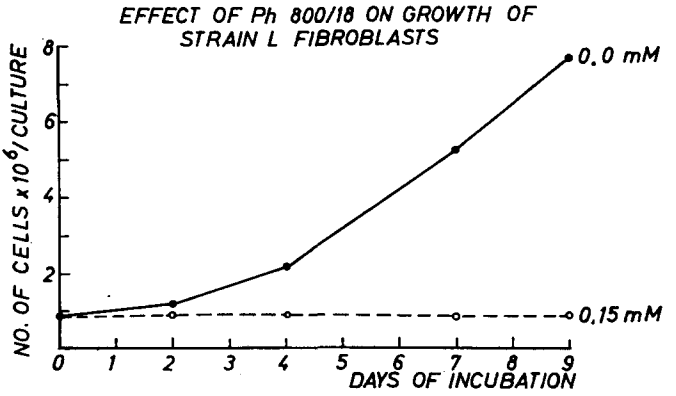


FIG. 10. Effect of Ph 800/18 on growth of strain L fibroblasts.

stored material. The initial cell number per culture was 0·75 × 10^6. After 5 days of incubation the cultures were trypsinized and the cell number counted. The average results, which are seen in Table 8 show that there was no Ph 800/18 activity in either the ascites fluid or the cell extract, while the activity in Tyrode's solution caused a complete growth inhibition. Thus, inactivation or removal from the peritoneal cavity seem to be more likely explanations of the inactivity of the ascites fluid from treated animals than binding to the tumour cells.

In order to test whether Ph 800/1 is inactivated by Ehrlich's ascites tumour and by a suspension of mouse liver cells, the compound was dissolved at a concentration of 0·2 mM in.: (1) Tyrode's solution, (2) ascites fluid containing 150 × 10^6 tumour cells per ml, and in (3) Tyrode's solution containing 300 mg per ml of mouse liver tissue cut into fine pieces. Tyrode's solution and cell suspensions without Ph 800/1

TABLE 8. ELIMINATION OF PH 800/18 FROM EHRLICH'S ASCITES TUMOUR IN 1 HR
(Cytostatic effect of ascites and cell extract on L-strain fibroblasts)

Supplement to culture medium	No. of cells × 10^6 per flask*	
	Initial	After 5 days
10% Tyrode's sol. without Ph 800/18	0·75	8·83
10% Tyrode's sol. with 0·5 mM Ph 800/18	0·75	0·69
10% ascites from non-treated mice	0·75	7·44
10% ascites withdrawn from mice 1 hr after injection of 0·5 m-moles Ph 800/18 per kg body weight	0·75	7·63
10% cell extract from non-treated mice	0·75	6·77
10% cell extract from mice 1 hr after injection of 0·5 m-moles Ph 800/18 per kg body weight	0·75	8·23

* Average of from three to six cultures.

TABLE 9. INACTIVATION OF PH 800/1 *in vitro* BY EHRLICH'S ASCITES TUMOUR AND MOUSE LIVER
(Cytostatic effect on L-strain fibroblasts of Ph 800/1 preincubated in Ehrlich's ascites tumour and mouse liver cell suspension)

Preincubation mixture	Supplement to culture medium	No. of cells × 10^6 per flask*	
		Initial	After 5 days
—	—	1·4	4·18
—	Ph 800/18 (0·02 mM)	1·4	0·96
Tyrode's sol.	10% Tyrode's sol.	1·4	5·17
Tyrode's sol. with 0·2 mM Ph 800/18	10% Tyrode's sol. with Ph 800/18	1·4	0·97
Ascites tumour	10% ascites	1·4	8·09
Ascites tumour with 0·2 mM Ph 800/18	10% ascites with Ph 800/18	1·4	2·56
Liver cell suspension	10% supernatant	1·4	6·08
Liver cell suspension with 0·2 mM Ph 800/18	10% supernatant with Ph 800/18	1·4	0·66

* Average of three to four cultures.

served as control material. The solutions and the suspensions were incubated at 37 °C under permanent rotation. After 1 hr the cell suspensions were centrifuged, and the supernatant together with the Tyrode's solutions were stored at 3 °C. The biological test was carried out with the L cells as described above. The figures shown in Table 9 are average values of from three to four cultures.

It appears from the table that incubation *in vitro* for 1 hr at 37 °C in Tyrode's solution and in the liver cell suspension did not influence the Ph 800/1 activity. Incubation with the ascites tumour caused a slight decrease in Ph 800/1 activity, but too small to explain the almost complete disappearance in less than 1 hr under *in vivo* conditions. Thus, removal from the peritoneal cavity seem to be a more likely explanation of the inactivity of the ascites fluid from treated animals than inactivation of the compound.

DISCUSSION

During recent years several authors have reported on the biological effects of lipoic acid, which is known to function as a coenzyme in the oxidative decarboxylation of α-keto acids. Thus, a profound effect on embryonic development in sea urchins,[7] and a considerable growth inhibition of *Allomyces macrogynus*[8] has been described. Lipoic acid and similar cyclic disulphides were also found to be potent inhibitors of regeneration in *Hydra* and *Planaria*,[9–11] and a clear antimitotic action on onion roots was demonstrated by Di Carlo.[12] The latter observation led to a study of the antineoplastic potentialities of lipoic acid,[13–15] but no tumour inhibiting activity could be demonstrated.

As outlined by Schotte and Nygård[1] there are good reasons to believe that the biological effects are due to the five-membered disulphide ring, and in agreement with this assumption Machlis[8] demonstrated an inactivation of 6:8-dithio-octanoic acid (lipoic acid) when the five-membered disulphide ring was replaced by the six-membered disulphide ring in 5:8-dithio-octanoic acid. The biological effect of the disulphide ring is, however, not only determined by the number of carbon atoms in the ring but also by that of the side chain. Thus, studying the effect of lipoic acid derivatives on malic dehydrogenase activity and regeneration of *Planaria*, Henderson and Eakin[16] found approximately a fourfold increase in the inhibitor activity for each increase of one carbon atom in the side chain.

The results of the present investigation indicate that the attachment of the side chain to the ring at the third position is not essential. Compounds with side chains at the fourth and fifth position also showed biological activity, in some cases even more so than lipoic acid. The introduction of a semicarbazone chain at the fourth position (Ph 800/1 and Ph 800/18) made the compound particularly potent, but also 2:3-dithio-4:5-spirodecane (Ph 800/2) and 1:2-dithiolane-4-carbonic acid (Ph 800/21) showed greater activity than lipoic acid at equimolar concentration. Whether the introduction of longer side chains at the fourth position will yield compounds with greater growth inhibiting potentialities remains to be shown.

The mechanism of the cytostatic effect of lipoic acid in *Hydra* and *Planaria* has been studied by Henderson and Eakin,[16] who demonstrated an inhibition of several dehydrogenases. However, since malic dehydrogenase was much more sensitive than any other enzymes, the inhibition of this enzyme was believed to be the basic factor involved in the biological effect. In further support of this conclusion Henderson and Eakin point out that the presence of oxalacetate will protect regenerating systems towards the deleterious effects of cyclic disulphides. Wiskich and Morton[17] have studied the effects of lipoic acid on the respiration and oxidative phosphorylation of mitochondria isolated from beetroot. Besides an inhibition of the respiratory chain oxidation of reduced coenzyme I, an uncoupling of oxidative phosphorylation by

lipoate was demonstrated by these authors, who suggested that inhibition of primary dehydrogenase as well as uncoupling of oxidative phosphorylation may be due to exchange reactions with essential sulphydryl groups.

In the present experiments a considerable anti-respiratory effect on Ehrlich's ascites tumour cells was demonstrated, while strain L fibroblasts seemed more resistent. This difference did not parallel the differences in cytostatic effects on these two types of cells. Therefore, at the present moment it seems impossible to decide whether the cytostatic activity observed in this investigation is due to an inhibition of the respiratory metabolism.

The apparent resistance of Ehrlich's ascites tumour cells towards the cytostatic effects of 1:2-dithiolane derivatives seem mainly to be due to their rapid elimination. This represents a serious limitation to the use of these substances in the treatment of neoplastic disorders. If this problem could be solved, 1:2-dithiolane derivatives may prove to be useful drugs in cancer chemotherapy. It should, however, be remembered that if the effect of these compounds is based on an inhibition of the respiratory metabolism, then a combination with an antiglycolytic therapy may be necessary to obtain a complete cytostatic effect.

Acknowledgement—Thanks are due to Pharmacia, Uppsala, for generous support.

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