first converted into 1:2-dihydro-1:2-epoxynaphthalene, then this difference may be due to the relative reactivities of this epoxide and 1:2-epoxy-1:2:3:4-tetrahydronaphthalene.

Instances of the formation of epoxides *in vivo* from compounds containing isolated double bonds have been reported previously. Davidow & Radomski (1953), for example, found that the insecticide, Heptachlor, was converted into the epoxide by dogs and rats and the epoxylation of certain steroid double bonds by micro-organisms is also known (Shull & Bloom, 1958). These epoxides are, however, unusually stable.

SUMMARY

1. The preparation of *S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)glutathione (I) is described.

2. 1:2-Dihydronaphthalene was converted by rat-liver slices into the glutathione derivative (I), which, with rat-kidney homogenate, yielded *S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-L-cysteine (II) and with rat-kidney slices yielded *N*-acetyl-*S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)-L-cysteine (III).

3. *trans*-1:2-Dihydroxy-1:2:3:4-tetrahydronaphthalene and 2-naphthol were also formed by the action of rat-liver slices on 1:2-dihydronaphthalene.

4. A rat-liver microsomal system converted 1:2-dihydronaphthalene into *trans*-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene, and, in the presence of glutathione, cysteine or *N*-acetylcysteine, to the respective *S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl) derivatives (I), (II) or (III). The formation of the glutathione derivative (I), but not of the derivatives (II) and (III), was increased by the addition of supernatant from the preparation of the microsomes.

5. The addition of glutathione to 1:2-epoxy-

1:2:3:4-tetrahydronaphthalene was increased in the presence of the supernatant fraction of rat liver, whereas the addition of cysteine and *N*-acetylcysteine to the epoxide was not affected.

6. The supernatant fraction of a rat-liver homogenate in 0.1M-phosphate buffer, pH 7.4, appears to contain an enzyme which activates glutathione.

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Biochemical Studies with Methanesulphonyl Esters, Especially β -Chloroethyl Methanesulphonate

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Methanesulphonyl esters are alkylating agents (Ross, 1953), and are used as anti-neoplastic agents. Haddow & Timmis (1951, 1953) reported that

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several $\alpha\omega$ -dimethanesulphonoxy glycols inhibited the growth of the Walker 256 rat carcinoma, 1:4-dimethanesulphonoxy-*n*-butane (Myleran, busulphan) and 1:5-dimethanesulphonoxy-*n*-pentane being most active. Elson (1955, 1957, 1958*a, b*)

and Elson, Galton & Till (1958) have shown that these compounds inhibit cell division in rat bone-marrow. Doses may be given that selectively depress the neutrophil count in the blood. Again, as in the Walker-tumour-inhibition test, busulphan and 1:5-dimethanesulphonoxy-*n*-pentane show the highest activity in depressing the neutrophil count. Physicochemical experiments that may explain their higher activity compared with other members of the series have been reported by Hudson, Timmis & Marshall (1958).

Busulphan has become an accepted palliative agent for treating chronic myeloid leukaemia (Galton, 1956). 1:4-Dimethanesulphonoxy-1:4-dimethyl-*n*-butane (dimethyl busulphan) is chemically more reactive than busulphan, and acts more rapidly than it in depressing the number of circulating neutrophils (Elson, 1957), but is only a weak inhibitor of growth of Walker carcinoma (Hudson & Timmis, 1955; Timmis & Hudson, 1958).

1:6-Dimethanesulphonoxy-mannitol (mannitol busulphan) is a very soluble, non-toxic compound that inhibits the growth of the Walker carcinoma more than bone-marrow growth when administered to rats (Haddow, Timmis, Leese, Lister & Brown (1957); Haddow, Timmis & Brown (1958); Vargha & Kuzsman, 1959). Clinical trials with this compound have not yet been reported in detail. Haddow & Ross (1956) report that ethyl methanesulphonate (CB 1528) and β -chloroethyl methanesulphonate (CB 1506) inhibit growth of Walker carcinoma. Other experimental anti-neoplastic studies with β -chloroethyl methanesulphonate have been reported by Consoli, Ricciardi, Talarico & Altucci (1957*a, b*), although clinical trials with this compound have been disappointing (Papac, Galton, Till & Wiltshaw, 1958).

Roberts & Warwick (1958) have shown that a substantial part of a dose of ethyl methanesulphonate administered to rats appears as *S*-ethyl-*N*-acetyl-L-cysteine and derivatives in the urine. They have also demonstrated (Roberts & Warwick, 1959) that rats probably metabolize most of a dose of busulphan via a cyclic tetrahydrothiophenium ion, showing that *in vivo* some thiol groups are alkylated by methanesulphonyl esters.

There are few reports on the effects of methanesulphonyl esters upon the activities of enzymes containing essential thiol groups. Holzer (1956) reported that 0.8 mM-busulphan caused 50% inhibition of crystalline rabbit-muscle triose-phosphate dehydrogenase *in vitro*.

Ebina & Kurosu (1958) studied the effects of 1:9-dimethanesulphonoxy-nonane ('nonane') on the respiration and glycolysis of three ascites tumours *in vitro*. Respiration was inhibited, anaerobic glycolysis unaffected and aerobic glycolysis stimulated by this compound.

The work reported here represents an attempt to increase knowledge and understanding of the biological effects of methanesulphonyl esters.

EXPERIMENTAL

Methods

Ehrlich ascites-tumour cells were grown intraperitoneally in inbred black C. 57 mice. Tumour fluid was diluted with approx. 10 vol. of 0.95% NaCl and the cells were separated by low-speed centrifuging. The tumour cells were separated from ascitic plasma and erythrocyte contamination by centrifuging (approx. 200 *g* for 1½ min.) from four or five successive samples of 0.95% NaCl, and were suspended in a Ringer solution for use. Dry-weight determinations were by (a) packed-cell volume, estimated in Wintrobe tubes, centrifuged for 54 000 *g*-min. from Krebs-Ringer phosphate (Umbreit, Burris & Stauffer, 1949); 186 mg. dry wt./ml. of packed cells; or (b) by weighing a dried pellet of cells in a tared centrifuge tube.

In manometry, Krebs-Ringer phosphate, and Krebs-Ringer bicarbonate (Umbreit *et al.* 1949) were used for respiration and anaerobic glycolysis measurements respectively, with standard techniques in the Warburg apparatus, at 37.5°. Metabolic quotients were calculated from tangents to reaction progress curves, or between the time intervals stated.

Spectrophotometric experiments were carried out at room temperature, 20–22°, in silica cuvettes of 1 cm. light path, in a Unicam SP 500 spectrophotometer. Hydrogen-ion concentrations were measured with the glass electrode.

Materials

Unless otherwise stated, all chemicals were of A.R. quality. Sodium pyruvate was prepared from fractionally redistilled pyruvic acid at 0°, care being taken to prevent the solution becoming alkaline during neutralization. Other chemicals were: L-alanine, L-aspartate, thiamine pyrophosphate from Roche Products Ltd.; L-malic acid, oxaloacetic acid, *cis*-aconitic acid and DL-isocitric lactone from California Foundation for Biochemical Research; oxidized and reduced diphosphopyridine nucleotide (DPN and DPNH) and cytochrome *c* from Sigma Chemical Co.; thiocetic acid from L. Light and Co. Ltd.; α -oxoglutaric acid and DL-malic acid from British Drug Houses Ltd. Enzymes were prepared and assayed as detailed below.

Enzymes

Succinic dehydrogenase. This was assayed manometrically with phenazine methosulphate as the hydrogen acceptor, in a 10 mM-phosphate (pH 7.2) extract of an acetone-dried powder of rat liver (Singer & Kearney, 1957).

Succinoxidase-cytochrome c reductase. The enzyme in a 0.3 M-sucrose homogenate of rat liver was assayed by measuring the reduction of cytochrome *c* spectrophotometrically, at 550 m μ , in the presence of HCN (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955).

Succinoxidase. The activity of cell-clumps centrifuged from a brei of rat liver prepared in 0.95% NaCl was estimated manometrically.

Fumarase. This enzyme was partly purified from a mitochondrial fraction of rat liver (prepared in 0.25 M-sucrose), according to Massey (1955). Activity was measured by

following the changes in fumarate concentration spectrophotometrically at 240 $m\mu$ (Racker, 1950).

L-Malic dehydrogenase. This was prepared from an acetone-dried powder of rat-liver mitochondria to the stage of the first calcium phosphate precipitation, and assayed according to Ochoa (1955).

Lactic dehydrogenase. The fraction of a water homogenate of Ehrlich ascites-tumour cells, precipitated between 0.5 and 0.75 saturation with $(\text{NH}_4)_2\text{SO}_4$ at 0° , was used. The enzyme was also partly purified from rat muscle and assayed according to Kornberg (1955).

Aconitase. Activity in the malic-dehydrogenase concentrate was measured by following the decrease in *cis*-aconitate at 240 $m\mu$ (Racker, 1950).

L-Glutamic dehydrogenase was assayed spectrophotometrically by measuring DPN reduction, in a 5 mm-phosphate extract (pH 7.2) of an acetone-dried powder of rat-liver mitochondria (Strecker, 1955).

Pyruvic-dismutation system. A rat-liver homogenate prepared in 0.3 M-sucrose containing 2 mm-ethylenediaminetetra-acetate (EDTA), pH 7.0, was centrifuged free of whole cells and nuclei at 0° . Lactic dehydrogenase, co-factors, pyruvate and phosphate or bicarbonate buffer, pH 7.0, were added. Full conditions are given in Table 7.

RESULTS

Effects in vitro of methanesulphonyl esters on the anaerobic glycolysis of Ehrlich ascites-tumour cells

Incubation of Ehrlich ascites-tumour cells for 2 hr. at 37.5° with 10 mm- β -chloroethyl methanesulphonate (with or without 10 mm-nicotinamide), 10 mm-1:6-dimethanesulphonoxymannitol or 10 mm busulphan (saturated suspension) did not alter the glycolysis rates of these cells, washed free of drug, relative to controls.

Anaerobic glycolysis measured in the presence of large quantities of β -chloroethyl methanesulphonate, and corrected for acid production from drug hydrolysis, was initially stimulated but later fell (Table 1).

Effects in vitro of methanesulphonyl esters on the respiration of Ehrlich ascites-tumour cells

The pure hydrolysis products of β -chloroethyl methanesulphonate (5 mm- β -chloroethanol, 5 mm-sodium methanesulphonate) do not affect the respiration of ascites-tumour cells. With all the esters studied, except dimethyl busulphan, less than 1% was hydrolysed in a 2 hr. incubation period, at 37.5° .

β -Chloroethyl methanesulphonate and other methanesulphonyl esters inhibited the respiration of ascites-tumour cells, the degree of inhibition, for a given concentration of drug, depending upon the substrate being oxidized (Tables 2 and 4). Attempts to protect the respiration of ascites-tumour cells against β -chloroethyl methanesulphonate, or to reverse the inhibition of respiration with thioctic acid, nicotinamide or thiamine, were unsuccessful.

L-Cysteine gave complicated results (Fig. 1), which were interpreted to mean that this compound does not protect the respiration of ascites-tumour cells from β -chloroethyl methanesulphonate, but does cause an initial increase in oxygen uptake due to auto-oxidation and an inhibition of cell respiration, probably due to the destruction of a respiratory factor (Slater, 1949).

The inhibition of ascites-cell respiration increases with time only at high concentrations of β -chloroethyl methanesulphonate. Graphs of respiratory inhibition plotted against time are linear when plotted on log log co-ordinates, for a given drug concentration, when ascites cells are oxidizing added succinate or pyruvate (Fig. 2). When the cells are oxidizing endogenous substrates only, β -chloroethyl methanesulphonate inhibits respiration, but this inhibition remains constant or decreases with time.

Graphs of inhibition of respiration of ascites-tumour cells oxidizing succinate or pyruvate,

Table 1. *Anaerobic glycolysis of Ehrlich ascites-tumour cells in the presence of varying quantities of β -chloroethyl methanesulphonate*

Cell suspension was added from the side arms of the manometer vessels at zero time. Results were calculated from tangents to the CO_2 -evolution progress curves corrected for acid production from drug hydrolysis and tissue alkylation. Each flask contained Krebs-Ringer bicarbonate solution, pH 7.3, 36 mm-glucose, 2.50 mg. dry wt. of ascites cells in a total volume of 3.3 ml. The gas phase was $\text{N}_2 + \text{CO}_2$ (95:5) and the temperature was 37.5° . Acid production from 128 mm- β -chloroethyl methanesulphonate, in the presence of boiled cells, was 13 μl . of CO_2 /hr.

Time from beginning of experiment (min.)	β -Chloroethyl methanesulphonate, initial concentration (mm)			
	0	43	85	128
	Q_0^{N} * values* and (% control)			
5	+41.5 (100%)	+43.4 (105%)	+48.7 (118%)	+55.8 (135%)
60	+41.5 (100%)	+43.5 (105%)	+48.7 (118%)	+23.9 (58%)
120	+41.5 (100%)	+43.4 (105%)	+21.9 (53%)	+17.5 (42%)
180	+36.7 (100%)	+38.7 (106%)	+7.0 (19%)	+5.2 (14%)

* For explanation of symbols see Dixon (1952).

Table 2. *Respiration of Ehrlich ascites-tumour cells incubated with methanesulphonyl esters as shown*

The inhibition of respiration in the presence of the drug and inhibition of respiration of cells washed free of drug are recorded. Warburg manometers contained the added substrates at a concentration of 20 mm, 0.1 ml. of 30% KOH on a filter-paper roll in the centre well and Krebs-Ringer phosphate (pH 7.4) to a total volume of 4 ml. The atmosphere was O₂ and the temperature 37.5°.

Drug	Substrates	Drug present			Cells washed free of drug		
		Dry wt. of cells (mg./vessel)	QO ₂ controls	Inhibition (%)	Dry wt. of cells (mg./vessel)	QO ₂ controls	Inhibition (%)
1:5-Dimethanesulphonoxy- <i>n</i> -pentane, 20.6 mm, 105 min.	Endogenous	25.0	-6.1	61	20.7	-7.0	2†
	Pyruvate	25.0	-8.5	81	20.7	-8.8	2†
	Succinate	—	—	—	20.7	-9.9	5
β -Chloroethyl methanesulphonate (CB 1506), 5 mm, 120 min.	Pyruvate	*Approx. 18	-9.3	15	13.6	-9.4	3†
	Oxaloacetate	*Approx. 18	-12.6	37	13.6	-12.0	0
	Pyruvate + oxaloacetate	—	—	—	13.6	-11.6	0
β -Chloroethyl methanesulphonate (CB 1506), 32.3 mm, 60 min.	Endogenous	23.1	-7.3	31	20.9	-6.9	0
	Pyruvate	18.8	-9.1	51	20.9	-9.3	0
	Succinate	28.1	-10.7	46	20.9	-10.9	0
β -Chloroethyl methanesulphonate (CB 1506), 56 mm, 105 min.	Endogenous	23.1	-6.4	62	13.9	-6.0	11
	Pyruvate	18.8	-9.3	85	13.9	-8.1	7
	Succinate	20.5	-10.4	70	13.9	-10.1	12

† Inhibition too small to be significant.

* Average of several determinations.

Fig. 1. Apparent respiration rates of Ehrlich ascites-tumour cells. Each Warburg flask contained 11.2 mg. of cells in 3.3 ml. of Krebs-Ringer phosphate containing 32 mm-sodium pyruvate, with 0.1 ml. of 30% (w/v) KOH on a filter-paper roll in the centre well. Additions: Δ , none; $\blacktriangle$, β -chloroethyl methanesulphonate, 19 mm; $\square$, L-cysteine, 5 mm; $\circ$, L-cysteine, 5 mm, and β -chloroethyl methanesulphonate, 19 mm. Results are calculated from tangents to the oxygen-uptake curves.

Fig. 2. Inhibition of respiration of Ehrlich ascites-tumour cells after different periods in various concentrations of β -chloroethyl methanesulphonate. Each flask contained 18.8 mg. of cells in Krebs-Ringer phosphate, with 25 mm-sodium pyruvate (---) or 20.4 mg. of cells and 20 mm-sodium succinate, (—). Results were calculated from graphs of oxygen uptake. Each vessel contained 0.1 ml. of 30% (w/v) KOH on a filter-paper roll in the centre well. Numbers on graphs represent the concn. of β -chloroethyl methanesulphonate as mm.

plotted against the logarithm of β -chloroethyl methanesulphonate concentration, are sigmoid in form. The same results, plotted as probit inhibition against log β -chloroethyl methanesulphonate concentration, produce straight lines at low concentrations of drug, but at higher drug concentrations the graphs curve upward, the upward curvature increasing with time as the degree of respiratory inhibition increases (Fig. 3). The concentration of β -chloroethyl methanesulphonate causing 50% inhibition of respiration decreases linearly with the logarithm of the experimental time. With β -chloroethyl methanesulphonate and 1:5-dimethanesulphonoxy-*n*-pentane, the inhibition of respiration of ascites cells is independent of pH in the range pH 5.0-7.4.

The inhibition of respiration of ascites-tumour cells by low concentrations of β -chloroethyl methanesulphonate is completely reversed by washing the cells free of drug, but with concentrations greater than approx. 30 mM the inhibition of respiration cannot be completely removed. It is above approx. 30 mM- β -chloroethyl methanesulphonate that the respiration of ascites cells is progressively inhibited, when pyruvate or succinate are the exogenous substrates of respiration (Fig. 2).

These facts indicate that β -chloroethyl methanesulphonate, and possibly other methanesulphonyl esters, inhibit the respiration of ascites-tumour cells by a physical effect. Unlike 2:4-dinitrophenol and other compounds that uncouple oxidative phosphorylation, β -chloroethyl methanesulphonate does not prevent glucose depressing the oxygen uptake (Crabtree effect) in ascites-tumour cells (Table 3).

Between 90 and 120 min. from the beginning of the experiment, the respiration *in vitro* of washed ascites cells oxidizing endogenous substrates is slightly inhibited in cells in contact with 5 mM- β -chloroethyl methanesulphonate (2% mean of 19 determinations). The addition of a number of

metabolites to suspensions of ascites-tumour cells raises the respiration rate by various amounts. β -Chloroethyl methanesulphonate (5 mM) inhibits the respiration of ascites cells to an extent related

Fig. 3. Inhibition of respiration of Ehrlich ascites-tumour cells by various concentrations of β -chloroethyl methanesulphonate (CB 1506), 120 min. after the addition of drug. Results, calculated from tangents to oxygen-uptake curves, are plotted on probit-log co-ordinates. Each vessel contained 15.3 mg. of cells in 4.0 ml. of Krebs-Ringer phosphate with 25 mM-sodium pyruvate, and 0.1 ml. of 30% (w/v) KOH on a filter-paper roll in the centre well.

Table 3. *Respiration of Ehrlich ascites-tumour cells in the presence and absence of glucose, β -chloroethyl methanesulphonate and 2:4-dinitrophenol*

Experiments were carried out in Warburg manometers in an atmosphere of O_2 at 37.5°. Each flask contained Krebs-Ringer phosphate to a final volume of 4.0 ml., 15.8 mg. dry wt. of cells and 0.1 ml. of 30% KOH on a filter-paper roll in the centre well.

Additions					
Glucose, 38 mM	-	-	+	+	+
β -Chloroethyl methanesulphonate (CB 1506), 5 mM	-	+	-	+	-
2:4-Dinitrophenol, 0.1 mM	-	-	-	-	+
Time interval (min.)	QO_2 values				
0-30	-10.8	-10.6	-6.6	-6.6	-9.9
30-60	-9.4	-8.9	-6.0	-6.0	-10.1
60-90	-8.3	-8.1	-5.4	-5.8	-9.0
90-120	-7.7	-7.4	-5.4	-5.4	-9.2

Fig. 4. Inhibition of respiration of Ehrlich ascites-tumour cells by 5 mM- β -chloroethyl methanesulphonate. Each vessel contained an average of 18 mg. of cells in 4.0 ml. of Krebs-Ringer phosphate; added substrates were initially 24 mM, adjusted to pH 7.0 with NaOH, except for oxaloacetate, where modified Ringer with 70 mM-phosphate buffer, pH 7.3, was used. Results are calculated from oxygen-uptake graphs between 90 and 120 min. from the addition of drug. In the centre well of each vessel there was 0.1 ml. of 30% (w/v) KOH on a filter-paper roll. Substrates used (numbers in parentheses show the number of determinations averaged for the point shown): 1, oxaloacetate (3); 2, pyruvate (3); 3, L-alanine (4); 4, succinate (5); 5, DL-lactate (4); 6, L-glutamate (4); 7, L-aspartate (5); 8, citrate (4); 9, fumarate (4); 10, α -oxo-glutarate (4); 11, DL-malate (4); 12, cis-aconitate (3); 13, endogenous substrates (19); 14, DL-isocitrate (2); 15, DL-methionine (2).

Table 4. Inhibition of respiration of Ehrlich ascites-tumour cells caused by 30 mM- β -chloroethyl methanesulphonate

Results were calculated between 90 and 120 min. from time the drug was added to cells in Warburg manometers. Each vessel contained Krebs-Ringer phosphate to a final fluid volume of 4.0 ml., an average of about 15 mg. dry wt. of cells and 0.1 ml. of 30% KOH on a filter-paper roll in the centre well. Incubation was at 37.5° in O_2 . The tumours were the second or third passage from the tumour bank and were growing slowly. The Q_{O_2} values were below normal.

Substrate	Q_{O_2} (controls)	Inhibition (%)	No. of determinations
Glucose (75 mM)	-3.5	46	(3)
Endogenous	-3.8	26	(4)
DL-Malate (30 mM)	-4.0	21	(1)
Citrate (30 mM)	-5.0	65	(2)
Pyruvate (30 mM)	-7.8	64	(3)
Succinate (30 mM)	-8.9	46	(3)

to the respiration rate (Fig. 4), but 30 mM- β -chloroethyl methanesulphonate does not inhibit respiration to this pattern (Table 4).

Metabolism of ascites-tumour cells from mice previously injected with methanesulphonyl esters

The respiration rates of ascites-tumour cells, oxidizing pyruvate or endogenous substrates, from mice injected intraperitoneally 4–4.5 hr. previously with 5 m-moles (/kg.) of ethyl methanesulphonate, β -chloroethyl methanesulphonate, 1:6-dimethanesulphonoxy mannitol, dimethyl busulphan or busulphan in iso-osmotic sodium chloride, or forced with 120 m-moles of busulphan/kg., 24 or 48 hr. before use, do not differ significantly from controls.

Enzyme experiments

Soluble succinic dehydrogenase was not affected by 10 mM- β -chloroethyl methanesulphonate, but was inhibited to a small variable extent by 1:5-dimethanesulphonoxy-*n*-pentane (25 mM), busulphan (2.3 mM), 1:6-dimethanesulphonoxy mannitol (37 mM), and a saturated suspension of dimethyl busulphan.

After a preincubation period of 2 hr., β -chloroethyl methanesulphonate inhibited a succinoxidase preparation when an excess of cytochrome *c* was added (Table 5).

A succinoxidase-cytochrome *c*-reductase preparation was inhibited by various methanesulphonyl esters (Table 6). The inhibition caused by β -chloroethyl methanesulphonate was investigated in more detail. Lineweaver & Burk (1934) plots showed 'mixed inhibition' curves (Fig. 5).

Enzymes not inhibited by incubation with 10 mM- β -chloroethyl methanesulphonate for 2 hr. at 37.5° or by various other methanesulphonyl esters were fumarase, L-malic dehydrogenase, lactic dehydrogenase, aconitase and L-glutamic dehydrogenase.

A pyruvic-dismutation system was variably inhibited by methanesulphonyl esters and a high concentration of diethyl ether (Table 7). Essentially identical results were obtained with bicarbonate-carbon dioxide or phosphate buffers.

DISCUSSION

The methanesulphonyl esters are alkylating agents that *in vivo* react with thiol groups (Roberts & Warwick, 1958, 1959). Of the thiol enzymes studied a pyruvic-dismutation system and a succinoxidase-cytochrome *c*-reductase system were inhibited. In both cases it is known that enzymic activity depends on the structural integrity of an enzymic complex, and that lipid solvents or other lipid-destroying agents are effective in disorganizing them.

Table 5. Succinoxidase activity of a rat-liver brei incubated with and without β -chloroethyl methanesulphonate and cytochrome c for various times

Succinate, final concn. 10 mM, was added from the side arm at the times shown. Experiments were carried out in Warburg manometers in O_2 at 37.5°. Each vessel contained 10.3 mM- β -chloroethyl methanesulphonate, 1.46 mg. dry wt. of liver, Krebs-Ringer phosphate to a final volume of 4.0 ml. and 0.1 ml. of 30% KOH on a filter-paper roll in the centre well.

Cytochrome c (0.05%)	β -Chloroethyl methanesulphonate (CB 1506), 10.3 mM	QO_2 (30 min.)	Inhibition (%)	QO_2 (120 min.)	Inhibition (%)
-	-	-68.9	.	-41.8, -45.9	.
-	+	-65.8	4*	-42.5, -41.8	3*
+	-	-72.3	.	-53.7, -50.7	.
+	+	-65.8	9	-42.5, -41.8	19

* Inhibition too small to be significant.

Table 6. Succinoxidase-cytochrome c-reductase activity of a rat-liver homogenate in the presence of varying amounts of methanesulphonyl esters

Rat liver, homogenized in 0.25M-sucrose + 2 mM-EDTA at pH 7.0 and 0°, was centrifuged free of whole cells and nuclei, diluted with EDTA solution and allowed to stand for 30 min. at 0°. Esters were in contact with the enzyme for 15 min. at room temp., 20°, before assay. Final concentrations: cytochrome c, approx. 40 μ M; potassium succinate, 2.5 mM, pH 7.0; phosphate buffer, 17 mM, pH 7.0; HCN, 0.3 mM; EDTA, pH 7.0, 0.85 mM. The assay was at room temp., 20°, in 1 cm. silica cuvettes at 550 m μ . Mean control rate of cytochrome c reduction, $+\Delta E_{550\text{ m}\mu}/10\text{ min.}$ was 0.527.

Ester	Concentration (mM)	Inhibition (% of control rate)
Busulphan	0.6	22
1:5-Dimethanesulphonyloxy-n-pentane	4.0	19
1:6-Dimethanesulphonyloxy mannitol	25.0	32
Ethyl methanesulphonate (CB 1528)	25.0	24

Fig. 5. Inhibition of rat-liver succinoxidase-cytochrome c reductase by β -chloroethyl methanesulphonate. Lineweaver & Burk (1934) plot of $1/v$ against $1/[S]$, where reaction velocity v is $+\Delta E_{550\text{ m}\mu}/10\text{ min.}$, $[S]$, succinate concentration (M). Drug was in contact with enzyme for 10 min. at room temp., 22°, before assay. Medium contained mM-EDTA and 16.6 mM-phosphate buffer, pH 7.0. Δ , Controls; $\blacktriangle$, 10.0 mM- β -chloroethyl methanesulphonate; $\square$, 25.1 mM- β -chloroethyl methanesulphonate.

In vitro, low concentrations of methanesulphonyl esters inhibited the respiration, but not the anaerobic glycolysis of ascites-tumour cells. The observed effects may be explained by physical actions of the compounds studied. This concept is supported, for β -chloroethyl methanesulphonate, by the complete reversibility, by washing, of respiratory inhibition with drug concentrations up to approx. 30 mM. At these concentrations, inhibition of respiration does not increase with time (Fig. 2); in fact inhibition seems to be instantaneously established by the presence of the drug.

No explanation is offered for the increased inhibition of respiration of ascites cells at elevated respiration rates, caused by different substrates, but data showing similar trends have been recorded elsewhere; for example, brain slices respiring in the presence of 0.08% of luminal or 3.3 mM-chloretone (Jowett & Quastel, 1937; Quastel, 1955). In some tissues the succinoxidase system is more resistant to drugs and physical damage than the DPNH-oxidase system. This may explain why 30 mM- β -chloroethyl methanesulphonate inhibits the respiration of ascites-tumour cells to a lesser

Table 7. *Pyruvate-dismutation system assayed in the presence and the absence of various methanesulphonyl esters*

Esters were in contact with enzymes for 45 min. at 37.5° before pyruvate was added from the side arm. Results were calculated from CO₂-evolution curves between 10 and 30 min. from tipping. Warburg manometers: total fluid volume, 3.0 ml.; atmosphere, N₂; final concentration of rat liver, 8% (homogenized in 0.3 M-sucrose + 2 mM-EDTA, pH 7.0, freed from whole cells and nuclei by centrifuging and saturated with CO₂); lactic dehydrogenase concentrate 1.0 ml. Final concentrations: sodium pyruvate, 46 mM; DPN, 2 mM; nicotinamide, 0.5 mM; thiamine pyrophosphate, 0.5 mM; MgCl₂, 0.5 mM; sodium fumarate, 0.3 mM; sucrose, 0.3 M; EDTA, 2 mM; potassium phosphate buffer, 10 mM, pH 7.0.

Ester	Activity of preparation (+ μ l. of CO ₂ /hr.)	Inhibition (%)
None (controls)	+130, +130	0
Busulphan (2.0 mM)	+120, +127	5
1:5-Dimethanesulphonoxy- <i>n</i> -pentane (10 mM)	+131, +124	2*
1:6-Dimethanesulphonoxy mannitol (10 mM)	+126, +126	3*
β -Chloroethyl methanesulphonate (CB 1506) (10 mM)	+112, +112	14
Diethyl ether (approx. 120 mM)	+101, +102	22

* Inhibition too small to be significant.

extent when they are oxidizing succinate than when they are oxidizing pyruvate or citrate. The progressive irreversible inhibition of ascites-tumour-cell respiration by high concentrations of β -chloroethyl methanesulphonate might be due to the disruption of a progressively greater proportion of the cells present.

SUMMARY

1. The respiration of Ehrlich ascites-tumour cells is inhibited by a variety of methanesulphonyl esters *in vitro*. This inhibition is reversed by washing the cells free of drug, reversal being complete at low drug concentrations.

2. Kinetic evidence and enzyme studies are presented which support the hypothesis that inhibition of respiration by β -chloroethyl methanesulphonate is due to physical effects of this ester on the hydrogen-transport chain of the cell.

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Preparation of Crystalline *Pseudomonas* Cytochrome *c*-551 and its General Properties

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A simple method has already been devised for extensive purification of four respiratory components from *Pseudomonas aeruginosa* in a true water-soluble state without the aid of detergents (Horio, 1958*a*). These components were called *Pseudomonas* (*P*)-cytochrome-554, *P*-cytochrome-551, *P*-blue protein and *P*-cytochrome oxidase (Horio, 1958*b*; Horio, Higashi, Matsubara *et al.* 1958). The same micro-organism also contains another cytochrome, *P*-cytochrome-560, which is similar to the so-called cytochrome *b*₁ and which has not yet been solubilized without the aid of sodium cholate (Horio, 1958*a*). From studies on purified respiratory components, cellular fragments and whole cells, Horio (1958*b*) and Yamanaka (1959) have concluded that in the electron-transferring system of *P. aeruginosa* *P*-cytochrome-551 and *P*-blue protein should be functional at a site corresponding to cytochrome *c* of animal mitochondria. Preliminary studies on chemical and physical properties of the purified components showed that *P*-cytochrome-551 bears a closer resemblance to a typical cytochrome *c* than it does to *P*-blue protein (Horio, 1958*a, b*).

The first crystallization of cytochrome *c* was accomplished by Bodo (1955), who isolated the

pigment from king penguin (*Aptenodytes patagonica*). It was extracted by the method of Keilin & Hartree (1937, 1945) and further purified with the use of the ion-exchange resin Amberlite IRC-50 (Margoliash, 1954*a, b*). By very similar methods, crystalline cytochrome *c* has since been obtained from a variety of sources, such as baker's yeast (Hagihara, Horio, Yamashita, Nozaki & Okunuki, 1956), ox heart (Kuby, Paléus, Paul & Theorell, 1956), horse heart (Nozaki, Yamanaka, Horio & Okunuki, 1957), fish heart (Hagihara *et al.* 1957) and wheat germ (Hagihara, Tagawa, Morikawa, Shin & Okunuki, 1958). In all cases, cytochrome *c* was purified from the crude to the purest state by chromatography on a weak cationic ion-exchange resin by procedures established by Paléus & Neilands (1950) and by Margoliash (1954*a, b*).

Despite the many similarities of *P*-cytochrome-551 to other types of cytochrome *c*, the standard procedure for the purification of the types of cytochrome *c* is inefficient for the purification of *P*-cytochrome-551 (Horio, 1958*a, b*). The main reason appears to be the acidic isoelectric point of *P*-cytochrome-551, which decreases the adsorptive power of a weak cationic resin such as Amberlite IRC-50 within its effective pH range.

In this paper, a suitable method for the preparation of crystalline *P*-cytochrome-551 from *P. aeruginosa* is presented together with a description of its general properties. A preliminary report on this work has been given by Horio, Higashi, Nakai, Kusai & Okunuki (1958).

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