BIOCHEMICAL BASIS OF CYTOTOXIC ACTIVITY OF SOME NEW N'-OXIDES OF N',N'-DIMETHYLAMINOALKYLAMIDES OF DODECANOIC ACID

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SUMMARY

The objective of the present investigation was to screen a series of new N'-oxides of N',N'-dimethylaminoalkylamides of dodecanoic acid for activity *in vitro* and to investigate the biochemical mode of action. On the basis of primary screening, one of the most active compounds, namely the N'-oxide of 10-(N',N'-dimethylamino-decyl)amide of dodecanoic acid (n=10) was chosen for detailed biochemical study. This compound inhibited the incorporation of ^{14}C-precursors (adenine, valine, thymidine, uridine) into appropriate macromolecules of P388 murine leukemia and Ehrlich ascites carcinoma cells. The amine oxide also interfered with energy-yielding processes (aerobic glycolysis, endogenous respiration). Cytotoxicity is a consequence of the cytolytic activity of the compounds mentioned above. Membranous effects were demonstrated by the measuring of the release of cytoplasmic materials absorbing at 260 and 280 nm, marker enzyme activities (LDH, MDH), release of protein from the cells into the culture medium, as well as by morphological examination. It is evident that the site of action of the amine oxides investigated was the biological membrane which, after interaction with the amine oxides, showed changes in molecular organization and osmotic and permeability characteristics.

KEY WORDS

N'-oxides of N',N'-dimethylaminoalkylamides of dodecanoic acid, Ehrlich and P388 cells, cytotoxicity, mode of action, cytolytic activity, morphological changes.

INTRODUCTION

Amine oxides represent a large group of compounds derived from tertiary amines containing a strongly polarized N-O bond /1,2/. A great number of amine oxides occurring in nature, or prepared synthetically, are biologically active compounds (antimetabolites and chemotherapeutics, psychotropic and cancerostatic compounds, etc.). Though some non-aromatic amine oxides have found wide industrial utilization due to their good surface active properties /3/, relatively little attention has been paid to their biological activity, in contrast to aromatic amine oxides /4,5/.

In view of the interesting chemical and biological activities shown by these compounds /6-9/, in the present study we investigated the cytotoxic activities and mode of action of N'-oxides of N',N'-dimethylaminoalkylamides of dodecanoic acid. The chemical structure of the substances studied is shown in Figure 1. Synthesis, properties and antimicrobial activity of the compounds have recently been described by Devinsky /7-9/. The compounds of this type belong to the so-called "soft" antimicrobially active compounds /8/.

The P388 murine leukemia cell-line is widely used both in experimental oncology and chemotherapeutic screening /10/. Ehrlich ascites tumour cells have been extensively used as an experimental model for biochemical investigation /11,12/. We have used Ehrlich cells also for the study of the mechanism of action of some antibiotics /13/, ethidium bromide /14/, isothiocyanates /15/ and other known cancerostatics /16/.

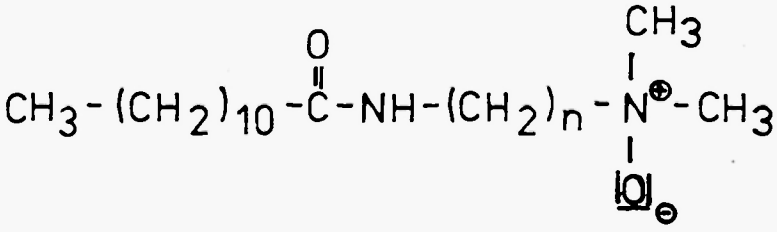

Fig. 1: The chemical structures of N'-oxides of N',N'-dimethylaminoalkylamides of dodecanoic acid (n=2 to 12).

MATERIALS AND METHODS

Ehrlich ascites carcinoma cells were maintained and propagated in albino mice, strain H (Institute of Experimental Pharmacology, Slovak Academy of Sciences, Dobra Voda, Czechoslovakia), about 10 weeks old and 20 to 25 g body weight, as described previously /17,18/. Ascitic plasma was poured off and an incidental layer of erythrocytes was removed /19/. The cells were suspended in Krebs-Ringer phosphate buffer, pH 7.4, without calcium but with ascitic serum (2.5%, v/v) and glucose (final concentration, 3.0 mmol/l). The number of cells was adjusted to $5x10^6$/ml of medium /18/. All operations were performed at 0-4°C. Mice with transplanted P388 cells were from Dr. V. Ujhazy, Cancer Research Institute, Bratislava. The cells were suspended in Krebs-Ringer phosphate buffer, pH 7.4, without calcium but with ascitic serum (2.5%, v/v) and glucose (final concentration, 3.0 mmol/l).

Materials

Chromatographically pure amine oxides were from the Department of Inorganic and Organic Chemistry, Faculty of Pharmacy, Komensky University, Bratislava. Substances were dissolved in Krebs-Ringer phosphate medium and/or in ethanol shortly before experiments. [8-^{14}C]Adenine sulphate (specific activity, 44 mCi/mmol), [U-^{14}C]valine (specific activity, 175 mCi/mmol), [2-^{14}C]thymidine (specific activity, 53 mCi/mmol), and [2-^{14}C]uridine (specific activity, 53 mCi/mmol) came from the Institute for Research, Production and Applications of Radioisotopes, Prague, Czechoslovakia. Other chemicals were supplied by Boehringer, Mannheim, Germany. The chemicals and enzymes necessary for the determination of glucose consumption and lactate formation, ATP, LDH and MDH activities, were purchased from Boehringer (Mannheim, Germany). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Primary biochemical screening (cytotoxicity assays)

In our laboratory, a new system has been developed and is being used routinely for mass screening of candidate compounds for antineoplastic activity /20-22/. The procedure used in evaluating the cytotoxic effect of the compounds was similar to that used when testing other metabolic inhibitors /21,23/. In short, cells were incubated 1 h in the presence of at least four selected concentrations of the substance, under defined conditions *in vitro*, and the active synthesis of nucleic acids and proteins was followed.

After 1 h of drug exposure, the test-tubes were transferred into an ice bath. [8-^{14}C]Adenine was added to the first series to a final concentration of 0.187 μCi per 1.02 μg and L[U-^{14}C]valine was added to the second series to a final concentration of 0.165 μCi per 2.64 μg. Both series were again incubated for 1 h at 37°C. In control experiments only Krebs-Ringer phosphate medium or ethanol were used. Incorporation was terminated by adding 1 ml of 5% TCA to each test-tube in an ice bath. The samples were filtered through synpor membrane filters, pore size 4 μm (Synthesia, Prague), the precipitate washed with 10 ml of cold 2.5% TCA and 10 ml water and dried at 105°C. The radioactivity was measured on a methane flow counter (Frieseke und Hoepfner, Erlangen, Germany).

Kinetics of DNA, RNA and protein synthesis

To define further the mechanism of action of selected compounds, the kinetics of DNA, RNA and protein synthesis inhibition were examined using isotope incorporation. This method has been described in detail /23/. The cells were incubated in a water bath at 37°C without shaking. At the indicated time intervals, samples of suspensions (1 ml) were analyzed for radioactivity in acid-insoluble material. Radioactivity was measured on a methane flow counter as in primary biochemical screening. In some cases, the nature of the labelled material was checked by alkaline-acid hydrolysis. In the case of adenine incorporation, 60.6% of the incorporated radioactivity corresponds to the RNA fraction and 39.4% corresponds to that of DNA. In the case of thymidine, 90% of its incorporation was found in DNA. In the case of uridine, 87.5% of the radioactivity was found in the RNA fraction /18/. All the data points are from duplicate determinations. The precision of these measurements is ±5%.

Glucose uptake and lactic acid production

The kinetics of aerobic glucose uptake and lactic acid production by both cell types were determined by commercially available tests (Boehringer, Mannheim, Germany) as described earlier /23/. The concentrations of glucose and lactate were determined enzymatically in the supernatant obtained after precipitation of suspensions of Ehrlich and P388 cells with 1 ml 0.6 mol/l glacial perchloric acid in an ice bath. The precision of these measurements is ±3%.

Respiration

The effect of amine oxide on endogenous respiration of both Ehrlich and P388 cells was determined on the basis of oxygen consumption in 154 mmol/l NaCl - 6.2 mmol/l KCl - 11 mmol/l sodium phosphate buffer, pH 7.4 /19/. Amine oxide was mixed with 2.0 ml of this medium, and 200 μl of the cell suspension in the same buffer solution was added. In control experiments, ethanol replaced the amine oxide solutions. The cellular respiration was measured with a Clark-type oxygen electrode in a thermostatically controlled reaction vessel equipped with a stirring device. The reaction system and procedure were essentially those described earlier /13,14/. Oxygen consumption was monitored for approximately 10 min and the linear portion of the oxygen consumption curve was utilized to calculate oxygen consumption rates. The respiratory rate was expressed as natoms of oxygen consumed per min and corresponding dry weight of the cells.

ATP determination

ATP level determination was carried out according to the method described in detail previously /24/. The decrease in NADH + H^+ absorbance was followed at 340 nm during 10 min. From the absorbance decrease per minute ΔA was calculated and after conversion, the ATP level was determined.

Protein determination

Protein concentration in the cell suspension was determined according to the method of Lowry *et al.* /25/.

Absorbance at 260 and 280 nm was followed by means of a spectrophotometer Spectromom 204, MON, Budapest, Hungary. Centrifuging of the cell suspension was performed using the Janetzki centrifuge K-24, Germany.

Determination of LDH and MDH activities

The activities of L-lactate: NAD^+ oxidoreductase (EC 1.1.1.27) and L-malate: NAD^+ oxidoreductase (EC 1.1.1.37) were determined spectrophotometrically in the supernatants of the cells at 340 nm (NADH + H^+) using commercial kits purchased from Boehringer Co., Mannheim, Germany. For details see /26/.

Morphological changes

These were studied using a native preparation at 1000x magnification by phase contrast microscopy.

RESULTS

Biochemical screening of cytotoxic activity

The results from primary biochemical screening of the cytotoxic activity on both Ehrlich ascites and P388 cells are summarized in Tables 1 and 2. For the chemical structures of the substances studied, see Figure 1. The inhibitory effect was characterized by IC_{50} values (molar concentration of compound required for 50% reduction of the incorporation rate). As is seen from the results in Tables 1 and 2, all the substances studied affected the incorporation of both precursors into the appropriate macromolecules of both cancer cells, in a concentration dependent manner. This has been confirmed not only by percentage inhibition (given in parentheses) but also by IC_{50} values. The lengthening of the joining chain in oxides of N',N'-dimethylaminoalkylamides of dodecanoic acid positively affected their cytotoxic activity in both cancer cells. Maximum activity was achieved with the compound No. 6 (length of the joining chain C = 10); further lengthening led to decrease in activity. Compound No. 6 was the most active of all derivatives of dodecanoic acid. Similar results were obtained by Devínsky *et al.* in the study of antimicrobial activity /27/. By comparing the IC_{50} values for both types of cells we can observe that IC_{50} values for adenine are higher than for valine, i.e., biosynthesis of proteins, indicated by the incorporation of [^{14}C]valine, is "more sensitive" than that of nucleic acids. However, it is interesting to point out that the IC_{50} values for both precursors are always lower for P388 than for Ehrlich cells.

On the basis of our previous results /20-23/, it is convenient to use an IC_{50}adenine:IC_{50}valine ratio (R), which is a suitable parameter to indicate the possible primary mode of action of the substance investigated. All ratios, as demonstrated in Tables 1 and 2, are in the range 0.98 to 2.75. Such ratios are typical also for other biologically active compounds which interfere with energy-generating systems in the cells. Inhibition of energy metabolism may be due to direct interaction or through the disorganization of membrane structure.

Effect on macromolecule biosynthesis

The values from biochemical screening represent the first

TABLE 1

Primary biochemical screening of N'-oxides of N',N'-dimethylaminoalkylamides of dodecanoic acid. The measure of the cytotoxic effect was the degree of inhibition of [^{14}C]adenine (a) and [^{14}C]valine (b) incorporation into TCA-insoluble fraction of Ehrlich ascites cells after 2 h incubation *in vitro*. The numbers represent cpm, with percentage of inhibition (or stimulation) in parentheses.

Substance No.	n	Formula M.W.		μmol/l 0	75	150	300	600	IC_{50} μmol/l	R
				Inhibition in cpm (percent inhibition in brackets)						
1	2	$C_{16}H_{34}N_2O_2$	(a)	2044(0)	2065(+1.02)	1736(15.07)	1604(21.53)	89(95.64)	417	1.48
		286.46	(b)	2502(0)	2157(13.79)	1765(29.46)	1168(53.32)	73(97.08)	280	
2	3	$C_{17}H_{36}N_2O_2$		2044(0)	2055(+0.54)	1985(2.89)	1503(26.33)	100(95.11)	405	1.09
		300.49		2502(0)	2292(8.39)	1927(22.98)	1605(35.85)	115(95.41)	370	
3	4	$C_{18}H_{38}N_2O_2$		2044(0)	2123(+3.86)	2060(+0.78)	1805(11.69)	98(95.2)	440	1.15
		314.52		2502(0)	2100(16.07)	1991(20.42)	1648(32.69)	98(96.08)	380	
4	6	$C_{20}H_{42}N_2O_2$		2044(0)	2060(+0.78)	1838(10.08)	319(84.39)	61(97.02)	235	1.42
		342.57		2502(0)	1946(22.22)	1354(45.88)	237(90.53)	72(97.12)	165	
5	8	$C_{22}H_{46}N_2O_2$		2044(0)	-	672(67.12)	101(95.06)	57(97.21)	< 150	?
		370.63		2502(0)	1636(34.61)	872(65.15)	72(97.12)	79(96.84)	113	
6	10	$C_{24}H_{50}N_2O_2$		2087(0)	634(69.62)	277(86.73)	219(89.51)	-	< 75	?
		398.68		1547(0)	197(87.46)	150(90.30)	140(91.08)	138(90.95)	< 75	
7	12	$C_{26}H_{54}N_2O_2$		2087(0)	1950(6.56)	1266(39.34)	1244(40.39)	844(59.56)	455	2.75
		426.73		1547(0)	981(36.59)	794(48.67)	547(64.64)	406(73.76)	165	

\+ stimulation over 100% against control sample. Substances Nos. 1 to 5 were dissolved in Krebs-Ringer phosphate medium and substances 6 and 7 in ethanol shortly before experiments. R = IC_{50} adenine:IC_{50} valine.

TABLE 2

Primary biochemical screening of N'-oxides of N',N-dimethylaminoalkylamides of dodecanoic acid. The measure of the cytotoxic effect was the degree of inhibition of [^{14}C]adenine (a) and [^{14}C]valine (b) incorporation into TCA-insoluble fraction of P388 cells after 2 h incubation *in vitro*. The numbers represent cpm, with percentage of inhibition (or stimulation) in parentheses.

Substance No.	n	Formula M.W.		μmol/l 0	75	150	300	600	IC_{50} μmol/l	R
				Inhibition in cpm (percent inhibition in brackets)						
1	2	$C_{16}H_{34}N_2O_2$	(a)	1868(0)	-	794(57.49)	903(51.66)	239(87.21)	145	1.12
		286.46	(b)	2349(0)	1540(34.44)	1040(55.73)	805(65.73)	128(94.55)	130	
2	3	$C_{17}H_{36}N_2O_2$		1868(0)	2631(+40.85)	1517(18.79)	742(60.28)	222(88.12)	265	0.98
		300.49		2349(0)	1716(26.96)	1490(36.57)	1104(53.00)	121(94.85)	270	
3	4	$C_{18}H_{38}N_2O_2$		1868(0)	1510(19.16)	1548(17.13)	326(82.55)	256(86.30)	225	1.73
		314.52		2349(0)	1648(29.84)	1040(55.73)	351(85.06)	151(93.57)	230	
4	6	$C_{20}H_{42}N_2O_2$		1868(0)	1553(16.86)	1516(18.84)	407(78.21)	222(88.12)	130	2.19
		342.57		2349(0)	1315(44.02)	898(62.03)	796(66.11)	213(90.93)	105	
5	8	$C_{22}H_{46}N_2O_2$		1452(0)	1457(+0.34)	988(31.96)	204(85.95)	195(86.57)	205	1.95
		370.63		2565(0)	1607(37.35)	709(72.36)	159(94.15)	130(94.93)	105	
6	10	$C_{24}H_{50}N_2O_2$		1452(0)	746(48.68)	318(78.10)	252(82.64)	205(85.88)	80	?
		398.68		2565(0)	563(78.05)	227(91.15)	142(94.46)	135(94.74)	< 75	
7	12	$C_{26}H_{54}N_2O_2$		1325(0)	1528(+14.87)	1324(+0.08)	1193(9.96)	769(41.96)	> 600	?
		426.73		1286(0)	862(32.07)	819(36.31)	664(48.37)	420(67.34)	325	

+ stimulation over 100% against control sample. Substances Nos. 1 to 5 were dissolved in Krebs-Ringer phosphate medium and substances 6 and 7 in ethanol shortly before experiments. R = IC_{50} adenine:IC_{50} valine.

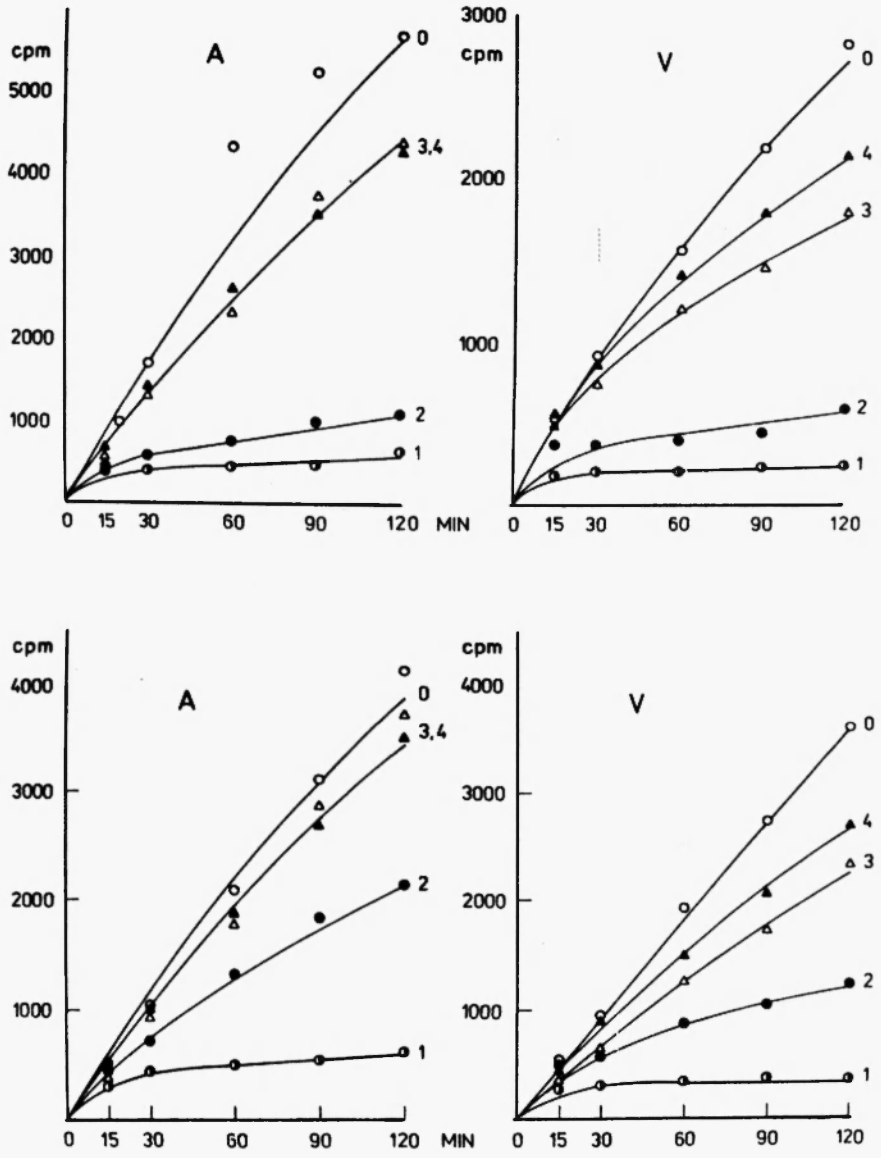

Fig. 2: The effect of compound No. 6 (Tables 1 and 2) on macromolecule synthesis of both Ehrlich ascites (top) and P388 (bottom) cells. Incorporation of radioactive adenine (A) and valine (V) into acid-insoluble fractions was determined by incubating cells with appropriate ^{14}C-precursors. Radioactive precursors and inhibitors were added to the cells at the same time. The test-tubes were incubated at 37°C, and 1 ml samples of each suspension were analysed for radioactivity in acid-insoluble material. The results are expressed as cpm/5x10^6 cells. Concentrations: 0 - none, 1 = 200 (◑), 2 = 100 (●), 3 = 50 (Δ), 4 = 25 (▲) μmol/l.

fundamental information about cytotoxic activity of new derivatives. In a first approach to determine the mode of action of the cytotoxically active compounds, the kinetics of DNA, RNA and protein synthesis inhibition were examined using isotope incorporation. Only when the time course is known is it possible to state at what time and concentration the inhibitory effects appear. Figure 2 demonstrates the inhibitory effect of compound No. 6 (Tables 1 and 2) upon the biosynthesis of macromolecules, indicated by incorporation of [^{14}C]adenine and [^{14}C]valine into TCA-insoluble material of both Ehrlich ascites (top) and P388 (bottom) cells. As can be seen from Fig. 2, compound No. 6 inhibited incorporation of both precursors into appropriate macromolecules of cancer cells; the extent of inhibition was dependent on the concentration of the compound in the incubation medium. At the highest concentration nearly complete inhibition of incorporation of both precursors occurred in both types of cancer cells. At the same time, the results appear to indicate that the inhibition occurs on addition of the drug to the cancer cell suspension, i.e., without a lag phase. Our results confirmed the data obtained in the biochemical screening.

As is known, [^{14}C]adenine is incorporated into both DNA and RNA. In order to differentiate which of the nucleic acids was more sensitive, experiments were carried out, the results of which are presented in Fig. 3. These results show that the amine oxide

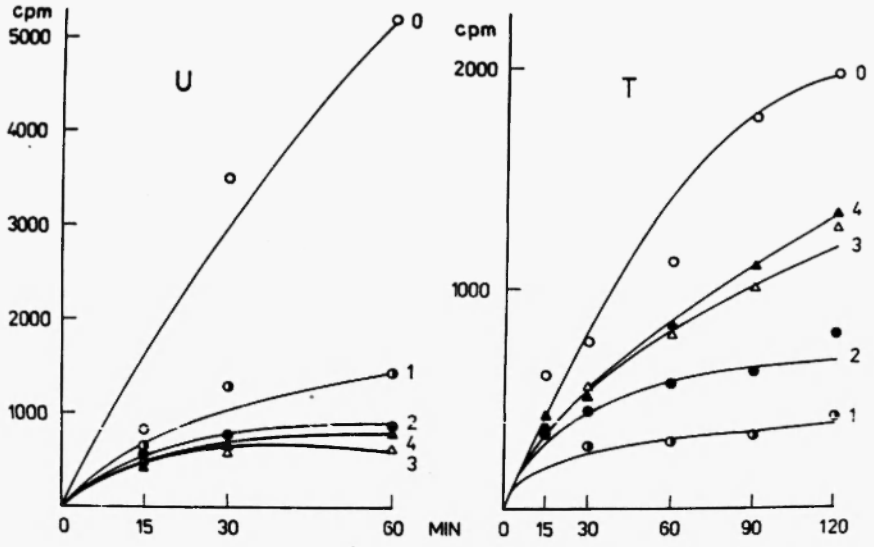

Fig. 3: The effect of compound No. 6 (Tables 1 and 2) on dynamics of [^{14}C]uridine (U) and [^{14}C]thymidine (T) incorporation into TCA-insoluble fractions of P388 cells. Other experimental conditions and symbols are the same as for Fig. 2.

inhibited incorporation of both precursors into appropriate macromolecules of P388 cells. Among the labelled isotopes tested, the incorporation of [^{14}C]uridine (RNA synthesis) was inhibited most strongly by the compound. At the highest concentrations tested (200, 100 and 50 μmol/l), nearly complete inhibition of RNA biosynthesis occurred. Even at the lowest concentration of amine oxide (25 μmol/l), significant inhibition of uridine incorporation occurred after 2 h incubation. The extent of thymidine inhibition (DNA synthesis) was dependent on the concentration of the compound in the incubation medium. Therefore, incorporation of [^{14}C]thymidine was inhibited proportionally to the concentration of the substance. However, it was less "sensitive" than that of uridine. The incorporation of all precursors was followed in the incubation medium containing glucose as a sole energy source.

Effects on energy-yielding processes

As macromolecular biosynthesis is an energy requiring process, we investigated further the action of compound No. 6 on some bioenergetic functions. Figure 4 demonstrates the effect of compound No. 6 (Tables 1 and 2) on aerobic glucose utilization (G) and lactic acid formation (L) by both Ehrlich ascites (top) and P388 cells (bottom). Both cancer cells consumed glucose from the medium linearly with time, even if its concentration fell by more than 50% of its original value. Likewise, a proportional increase in lactate concentration in the medium was seen in control cells. As shown in Fig. 4, none of the concentrations inhibited glucose consumption or lactate formation. On the contrary, the course of aerobic glycolysis was significantly stimulated, depending on concentration. There was practically no difference in the behaviour of the two cell types. The conversion of glucose to lactate in control cells was approximately 76% (calculated for the first 30 min of measuring glycolysis) which is in good agreement with our previous results, where the transformation of glucose into lactate was in the range of 75.0 to 83.5% /18/.

The amine oxide investigated significantly stimulated the aerobic glycolysis of both tumour cells (Fig. 4). Such a stimulation of glycolysis by amine oxide points to a potential interference with respiratory processes in cancer cells, or in isolated mitochondria, respectively /23/. In order to verify this, experiments were carried out, the results of which are presented in Fig. 5. Compound No. 6, at the lowest concentrations, first stimulated the endogenous respiration of the tumour cells, followed by linear inhibition of oxygen consumption in both types of cells. Both stimulation of respiration at low concentrations and inhibition of respiration at higher concentrations are typical for uncouplers of oxidative

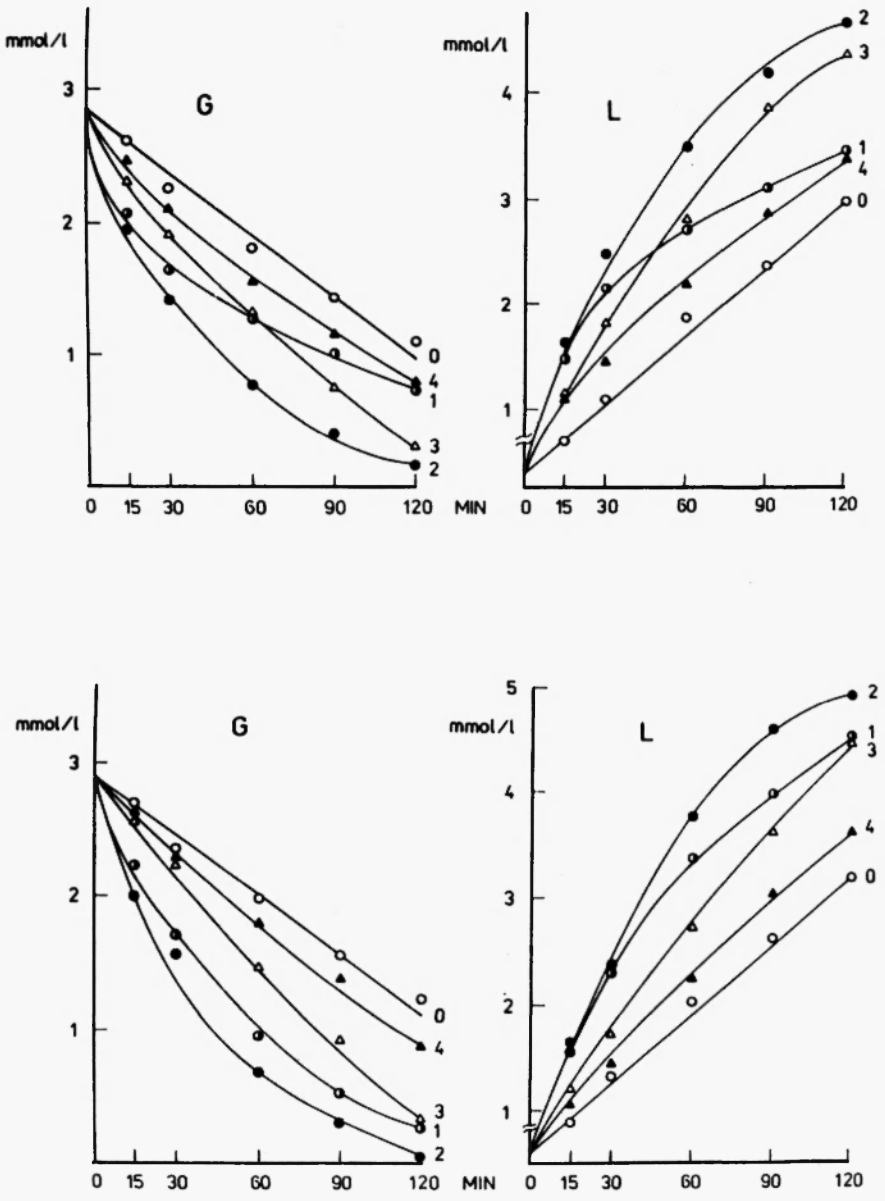

Fig. 4: The effect of compound No. 6 (Tables 1 and 2) on the kinetics of aerobic glucose utilization (G) and lactic acid formation (L) by both Ehrlich ascites (top) and P388 (bottom) cells. The cells were incubated at 37°C in the presence of different concentrations of compound No. 6. The initial glucose concentration was 3 mmol/l. At various times, 1-ml samples of suspension were analyzed for glucose and lactate. Other experimental conditions and symbols are the same as for Fig. 2.

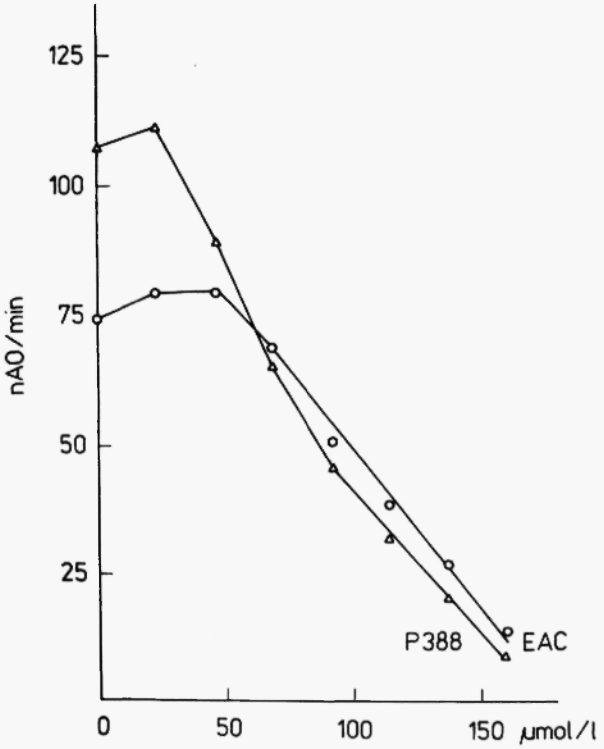

Fig. 5: The effects of compound No. 6 (Tables 1 and 2) on endogenous oxygen utilization by Ehrlich and P388 cells. Different concentrations of compound No. 6 were added to the respiring tumour cells. The ratio of oxygen consumption was determined immediately after the addition of compound No. 6 to the cells. Cell suspension (0.2 ml) containing 13.3 mg (Ehrlich) and 12.4 mg (P388) dry weight, was added to 2.0 ml of 0.9% NaCl solution-phosphate medium, pH 7.4. Oxygen uptake was measured at 30°C.

TABLE 3

The effect of compound No. 6 (Tables 1 and 2) on the level of ATP in P388 cells *in vitro*. The cells were incubated in the presence of different concentrations of the amine oxide studied.

Minutes of incubation	μmol/l				
	0	25	50	100	200
	nmoles of ATP/mg protein				
0 (0°C)	7.23	7.10 (1.8)[+]	6.25 (13.56)	5.82 (19.51)	2.55 (64.73)
120 (37°C)	7.37	7.20 (2.31)	6.12 (16.96)	5.55 (24.70)	1.72 (76.67)

[+]The numbers in parentheses represent percentage of inhibition.

phosphorylation. Though these results do not enable us to reach a definite conclusion about the exact mode of action, it is possible to state that the substance affects the respiratory processes of tumour cells and thereby also the production and/or utilization of ATP.

Effect on ATP level

To obtain direct evidence indicating interference of the drug with energy-generating systems, we studied the effect of amine oxide on ATP level in P388 cells. Table 3 shows that immediately after addition of amine oxide to the suspension of P388 cells in an ice bath, ATP decrease is proportional to the amine oxide concentrations tested. At the highest concentration, nearly 65% decrease in ATP level was observed. After 2 h incubation at 37°C the drop in the ATP level is much higher. However, the lowest concentration decreased the ATP level by only approximately 2%. The decrease in ATP level might be explained through the amine oxide's interaction with the processes related to oxidative phosphorylation, or indirectly through impairment of cell membrane integrity.

Biochemical evidence of cytolytic activity

The substance investigated showed a considerable effect on all the metabolic processes examined, especially at the highest concentrations utilized. We assumed, therefore, that the cytotoxic effect could be the consequence of cytolytic activity of the substance investigated. Membranous effects were demonstrated by several methods.

Cell injury resulting in increased plasma membrane permeability can be monitored by measuring the influx of extracellular molecules or the efflux of intracellular molecules to which the membrane would normally have restricted permeability. One of the most commonly used ways of determining the latter is by measuring the release of lactate dehydrogenase (LDH) or cytoplasmic enzymes from the cell into the extracellular environment /28,29/. Of course, also other intracellular constituents are considered, e.g., the loss of ions or cofactors (Ca^{++}, K^{+}, $NADH+H^{+}$), or release of material from prelabelled cells (e.g., ^{51}Cr).

Another indication of biological membrane damage is the release of cytoplasmic materials absorbing at 260 and 280 nm. At 260 nm, absorption is in particular by the purine and pyrimidine components of nucleic acids, while absorption at 280 nm is typical for proteins with Try, Trp and Phe amino acid components. A typical example is shown in Fig. 6. The results of such experiments

indicate that the cells incubated with amine oxides (25 to 200 μmol/l) for a longer time (2 h) released 260- and 280-nm absorbing materials, indicating damage of the treated cells (Fig. 6). Similar results were obtained also with Ehrlich cells (results not shown).

Another method of indicating the biological membrane damage is to monitor protein concentrations in both tumour cells and the culture medium (Table 4). From this Table it is clear that a significant release of protein occurred at concentrations of 25 μmol/l and higher, which is in agreement with Fig. 6. There is no detailed information on the nature of the cytoplasmic proteins released. Bystryn *et al.* /30/ found, in their study of the release of surface macromolecules from the cells of human melanoma and normal cells, that the release is not an artifact due to cell death. A significant quantity of released material had a molecular weight of 70,000-80,000, close to the weight of serum albumin.

One of the most commonly used methods characterizing cell injury due to increased plasma membrane permeability is the study of LDH activity in the extracellular medium /28/. This test is similar to that of the follow-up of viability (e.g., by staining) but it can be better quantified /31/. The enzyme plays an important role in the catabolism of glucose /32/.

The results of the study of the LDH activity as a typical "marker" of soluble (cytoplasmic) protein and MDH are presented in Tables 5 and 6. The results show a significant increase in activity of both enzymes, especially at the highest concentrations, in both cancer cells. At the highest concentration tested, the LDH activity increased about 6-fold (Ehrlich) and 4-fold (P388 cells). As for MDH, one MDH is present in cytoplasm and another in mitochondria. The examination of MDH activity thus indicates the release of proteins not only from the cytosol but also from mitochondria. Also the activity of MDH increased, by 6 (Ehrlich) and more than 4 (P388 cells) times. Another proof for injury of the biological membrane has thus been obtained.

The biochemical criteria complement the morphological changes which occurred in both Ehrlich ascites and P388 leukemia cells in the presence of the substance studied. Figure 7 shows the morphological changes of Ehrlich ascites cells induced by amine oxide. At the lower concentrations, gradual swelling of the cells is followed by shrinkage and bleb formation on the surface and, finally, at the highest concentrations, an actual disruption of the cells. Similar results were obtained also with P388 cells (results not shown).

DISCUSSION

The values from biochemical screening represent the first fundamental information about cytotoxic activity of new derivatives

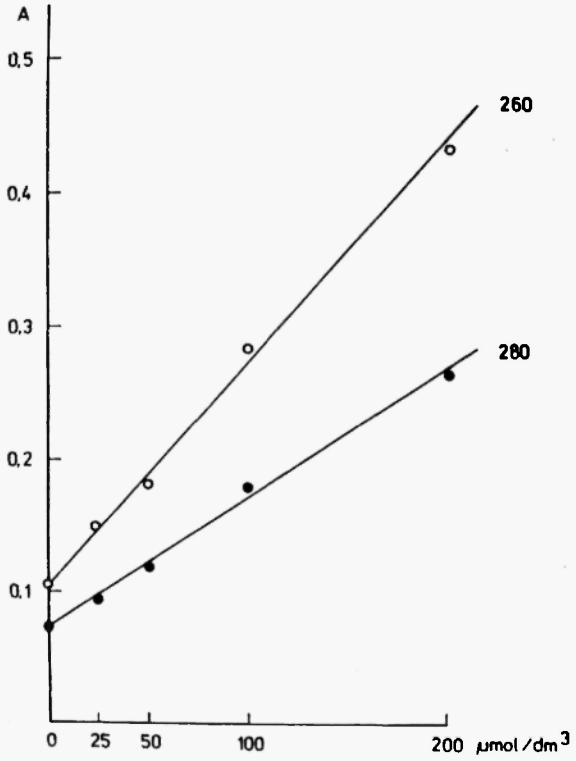

Fig. 6: Release of ultraviolet-absorbing materials from P388 cells treated with compound No. 6 (Tables 1 and 2) after 2 h incubation at 37°C.

TABLE 4

The effect of compound No. 6 (Tables 1 and 2) on release of proteins from Ehrlich carcinoma and P388 cells into culture medium after 2 h incubation

Cells		μmol/l				
		0	25	50	100	200
		mg proteins/ml				
Ehrlich	Supernatant	0.085	0.144	0.257	0.352	0.343
	Cells	1.072	0.874	0.832	0.783	0.750
P388	Supernatant	0.108	0.113	0.205	0.254	0.339
	Cells	1.083	0.940	0.850	0.837	0.756

Concentration of proteins in Ehrlich cells before incubation was 1.082 and in supernatant 0.035 mg/ml and for P388 cells 1.137 and in supernatant 0.094 mg/ml, respectively.

TABLE 5

The effect of compound No. 6 (Tables 1 and 2) on the activities of lactate and malate dehydrogenases (LDH and MDH), in the culture medium of Ehrlich ascites cells after incubation for 2 h.

Enzyme	μmol/l of the inhibitor				
	0	25	50	100	200
	μkat/l				
LDH	1.52	1.69	4.39	8.61	9.71
MDH	1.74	1.92	4.35	8.71	10.45

Activity of LDH before experiment was 0.42 and that of MDH 0.23 μkat/l.

TABLE 6

The effect of compound No. 6 (Tables 1 and 2) on the activities of lactate and malate dehydrogenases (LDH and MDH), in the culture medium of P388 cells after incubation for 2 h.

Enzyme	μmol/l of the inhibitor				
	0	25	50	100	200
	μkat/l				
LDH	1.69	1.61	2.53	5.06	7.60
MDH	1.48	1.57	2.18	5.92	6.53

Activity of LDH before experiment was 0.42 and that of MDH 0.61 μkat/l.

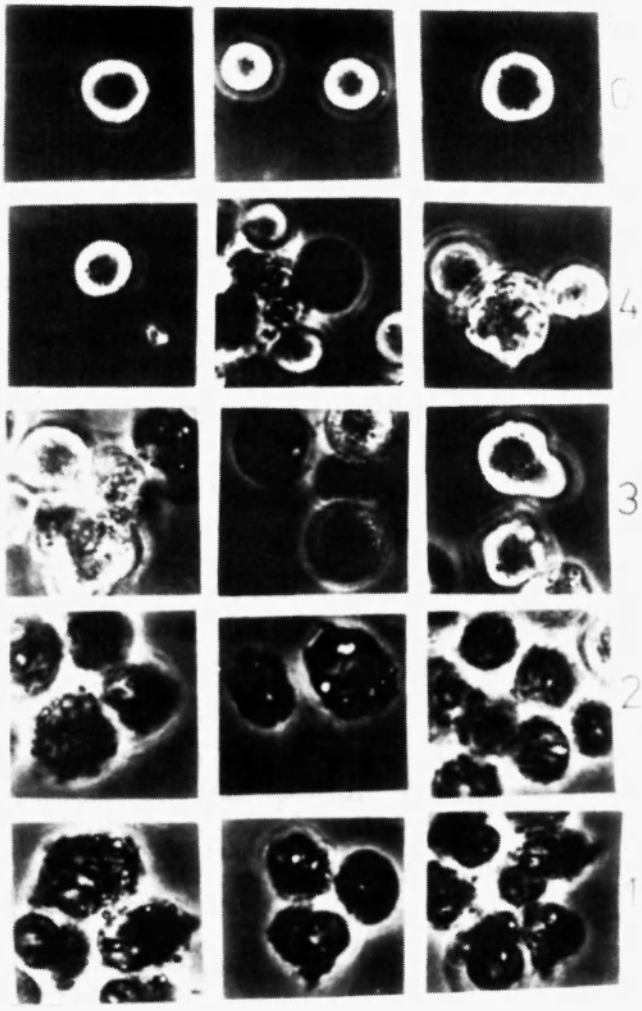

Fig. 7: Morphological changes in Ehrlich ascites cells induced by different concentrations of compound No. 6 (Tables 1 and 2) after 2 h incubation. 0 - none, 1 = 200, 2 = 100, 3 = 50, 4 = 25 μmol/l (1000x magnification).

of amine oxides. The data, which are obtained in a relatively short time, inform exactly whether the tested substance shows cytotoxic activity at all, and perhaps also indicate a possible mode of action. We have previously reported a rapid radiometric *in vitro* technique of primary screening for anticancer substances /20-22/. This method, which measures the drug-induced inhibition of [^{14}C]adenine and [^{14}C]valine incorporation, is relatively simple, reliable and sensitive. In tumour cells, the degree of influence on metabolic activity is identified by uniformly selected concentrations of the substances, in definite conditions *in vitro*, ensuring the active synthesis of proteins and nucleic acids.

From the results presented in Tables 1 and 2, it is evident that these new derivatives of amine oxides affected the incorporation of both precursors into appropriate macromolecules of both cancer cells, in a concentration dependent manner. Maximum activity was achieved with compound No. 6, namely the N'-oxide of 10-(N',N'-dimethylaminodecyl)amide of dodecanoic acid (n=10). The lengthening of the joining chain in oxides of N',N'-dimethylaminoalkylamides of dodecanoic acid positively affected their cytotoxic activity in both cancer cells. Further lengthening led to a decrease in activity. It is interesting to point out that the IC_{50} values for both precursors were always lower for P388 than Ehrlich cells. The use of P388 cells was not accidental, since, as demonstrated by Miyamoto and Terasaki /33/, these cells have different membrane composition. The differences in the behaviour of the two cell types can probably be attributed to the nature and composition of their cytoplasmic membrane. The ratios IC_{50} adenine:IC_{50} valine show the difference in the cytotoxicity of the substances, and they indicate primarily the similarity or diversity in the mode of action (in the initial changes). All ratios, as demonstrated in Tables 1 and 2, are in the range 0.98 to 2.75. Such ratios are typical for other biologically active compounds which interfere with generation or utilization of energy in cancer cells /14,16,22,23/. Volm /34/ found reasonably good correlation between a test based on the inhibition of radioactive nucleoside uptake and *in vivo* chemosensitivity of several rodent tumours.

Recently, Von Hoff *et al.* /35/ developed a radiometric system for the screening of antitumour agents. The index of cytotoxic effectiveness was based on the inhibition of transformation of [^{14}C]glucose into $^{14}CO_2$. This radiometric system (BAC-TEC 460) was optimalized with the aid of tumour cell lines of both human and animal origin. Scheithauser *et al.* /36/ used this new screening system for the selection of antitumour agents for the treatment of human colorectal tumours.

Although the mechanism of action of the amine oxide investigated has not been determined, our present results show that compound No. 6 inhibited incorporation of all 4 precursors (Figs. 2-3) into appropriate macromolecules of both tumour cells. This fact suggests that the effect of compound No. 6 lies at an underlying level of energy generation or transfer rather than at specific reactions in the biosynthesis of DNA and protein. The process of DNA synthesis is actually the culmination of many synthetic pathways. In the intact cell, interference with any of these pathways, as well as alterations and variation in the pool size of precursors, can alter the apparent rate of DNA synthesis and obscure specific drug effects. The rate of DNA synthesis is rapidly affected by the lowering of the level of any of the 4 deoxyribonucleotide triphosphates. Interference with the generation of high-energy phosphate bonds is one of the mechanisms available for induction of nucleotide deficiency. A depletion of nucleotide pools can serve as an efficient tool to inhibit cellular growth and to induce cell death under some circumstances.

As macromolecular biosynthesis is an energy requiring process, we investigated further the action of compound No. 6 on some bioenergetic functions. In recent years, a novel approach to the chemotherapeutic treatment of cancer has focused on the aberrant energy metabolism of neoplastic cells /37/. Preliminary results suggested that it would be possible to inhibit selectively the energy production, proliferation rate, and survival of tumour cells by affecting their specific enzyme systems /38/. Tumour cells show an increased utilization of anaerobic (glycolytic) metabolism for the production of cell energy /39/, and a direct correlation has been found between the growth of tumour cells and the rate of glucose utilization /40/. The most vigorously proliferating tumour cell lines produce as much as 50% of their total ATP from glycolytic metabolism /39/, with a corresponding decrease in oxidative phosphorylation and in cellular mitochondrial content /41/. Recently, Medina and Nunez de Castro /42/ have shown that when Ehrlich ascites cells can choose among different energy substrates (as is the case in physiological conditions), they choose glucose preferentially. Beckner *et al.* /43/ showed that the motility of metastatic cells in the human melanoma line A 2058 depends primarily on energy from glycolysis in the presence of glucose. These findings suggest that the inhibition of glycolysis *in vivo* might reduce the tumour cells' ability to leave the primary tumour mass and metastasize to secondary sites.

The amine oxide investigated significantly stimulated the aerobic glycolysis of both tumour cells (Fig. 4). Diamond *et al.* /44/

demonstrated that compounds like dinitrophenol (an uncoupler of oxidative phosphorylation) and oligomycin (an inhibitor of oxidative phosphorylation), which interfere with ATP synthesis, markedly stimulated lactate formation in intact noncycling 3T3 cells. Such a stimulation of glycolysis by amine oxide indicates its possible interference with respiration in cancer cells /23/, or in isolated mitochondria, respectively. Compound No. 6, at the lowest concentrations, first stimulates the endogenous respiration of tumour cells, followed by linear inhibition of oxygen consumption in both types of cells (Fig. 5). The inhibition of respiration can be due to the escape (release) of endogenous substrates from the intracellular space into the extracellular environment as a result of amine oxide interference with the integrity of biological membranes of these cells (Figs. 6, 7). Both stimulation of respiration at low concentrations and inhibition of respiration at higher concentrations are typical for uncouplers of oxidative phosphorylation. In these studies, the amine oxide had an effect similar to that of uncouplers of oxidative phosphorylation such as phenylhydrazonepropanedinitril and similar compounds, incorrectly named carbonylcyanidephenylhydrazones; they also had a considerable stimulatory effect on the aerobic glycolysis of Ehrlich cells /45/. Such cells are energy deficient due to the interference of the uncoupler with oxidative phosphorylation. It is well known that loss of ATP by diminished respiration is balanced by an approximately equal gain of ATP by an increased rate of lactate production /46/. Such an acceleration of primary metabolism resulting from activation of phosphofructokinase as the rate limiting enzyme of glycolysis by accumulated ADP is a regulatory phenomenon known also in tumour cells /41/. This assumption could be applied to lower concentrations when glycolytic ATP is capable of making up in some measure for ATP losses caused by the uncoupling effect of amine oxide. At the higher concentrations of amine oxide, probably due to an impairment of the integrity of biological membranes, such a compensation does not take place in a measure sufficient to maintain ATP at the required level.

To obtain direct evidence indicating interference of the drug with energy-generating systems, we studied the effect of amine oxide on ATP level in P388 cells (Table 3). The decrease in ATP level might be explained through the amine oxide's interaction with the processes related to oxidative phosphorylation, or indirectly through impairment of cell membrane integrity. In Ehrlich ascites cells, 80-90% of the total ATP-consumption has been accounted for by major processes: protein synthesis, proteolysis, Na^+/K^+-ATPase, Ca^{2+}-ATPase and RNA synthesis /47/. Although in the

case of many antineoplastic agents attention has been focused upon their effects on DNA, RNA and protein synthesis, the data of Hill /48/ emphasize that this is an over-simplification. Most agents have multiple effective target sites within the cells, and the primary cytotoxic events responsible for their clinical effectiveness remain to be elucidated. The work by Farber /49/ and others indicates that the inability to synthesize ATP in a cell leads to multiple secondary derangements in cellular metabolism.

The substance investigated showed a considerably inhibitory effect on all the metabolic processes examined, especially at the highest concentrations utilized. We assumed, therefore, that the cytotoxic effect could be the consequence of cytolytic activity of the amine oxide investigated. Membranous effects were demonstrated by several methods. The results of such experiments indicate that the cells incubated with amine oxide (25 to 200 μmol/l) for a longer time (2 h) released 260- and 280-nm absorbing materials, indicating damage of the treated cells (Fig. 6).

From Table 4 it follows that a significant release of protein occurs at concentrations 25 μmol/l and higher, which is in agreement with Fig. 6. Leakage of high molecular weight cytoplasmic constituents has been observed only after such cell membrane damage which leads as a rule to total cell destruction. Helenius and Simon /50/ ascribe this effect caused by some amphiphilic surface active substances to their solubilization effect; however, this is an oversimplified view.

Direct evidence characterizing cell injury due to increased plasma membrane permeability comes from the measurement of both lactate and malate dehydrogenase activities. The results in Tables 5 and 6 show a significant increase in activity of both enzymes, especially at the highest concentrations, in both cancer cells.

Monitoring LDH release is only one of many methods used to determine increases in plasma membrane permeability due to cell injury. Among others is the measurement of the release of a wide variety of intracellular enzymes, especially *in vivo*. Creatine phosphokinase has been frequently used, because of its specificity for muscle. However, creatine phosphokinase and other enzymes which have a dual location, such as glutamate-oxalacetate transaminase and malate dehydrogenase, release easily only their cytosolic isoenzymes and not the mitochondrial ones /51/. In addition, creatine phosphokinase is less stable in dilute solution than LDH /52/. Some enzymes associated with intracellular organelles are extremely resistant to release even in extreme conditions: for example, LDH is easily solubilized by detergents like Triton X-100, whereas peroxidase is highly resistant /53/. Mohr

and Emura /54/ state that the activity of glucose-6-phosphate dehydrogenase (G-6-PDH) released into the culture medium also seems to reflect adequately the degree of cell damage. They assert that the activity of G-6-PDH is a more sensitive indicator of cell damage than LDH.

From our results it is impossible to come to a conclusion about the mechanism by which the amine oxide investigated influences the tumour cell membrane integrity. Some authors suggest that the release of intracellular enzymes is related to the energy content of the cell, or with ATP decrease /55,56/. It seems that our findings are in agreement with this idea (Table 3).

As found recently /57,58/, the antimicrobials (1-methyldodecyl)dimethylamine oxide and (1-methyldodecyl)trimethylammonium bromide affect the cytoplasmic membrane of *E. coli*. The interaction results in release of intracellular material (K^+, 260 nm-absorbing material), an effect on dehydrogenase enzyme activity and inhibition of respiration. The final effect of both substances is the same; they differ only in their dynamics. Kopeckā-Leitmanova *et al.* /59/ summarized their results about the mode of action of quaternary ammonium salts and amine oxides upon bacteria determined *in vitro* in three stages. The first stage is characterised by the rate of onset of the action for which the polar interactions of molecules with bacterial membrane are responsible. In the second stage the polar and hydrophobic interactions are involved – this stage is the destructive effect on membranes. The third stage is represented by hydrophobic interactions which lead to death of cells.

It is evident that the site of action of the amine oxides investigated was the biological membrane which, after the interaction with amine oxides, showed changes in molecular organization and osmotic and permeability characteristics.

Many types of agents have been found to have membrane action even though they were originally designed to inhibit the synthesis or function of DNA /60/. New drugs have been synthesized which have lipophilic or membrane-selective structure and some of these are in early clinical trials /60/.

The surface membrane alterations which characterize the neoplastic transformation offer a potential for cytotoxic selectivity. Modification of the lipid and consequent physical properties of membranes has been shown to enhance the sensitivity of neoplastic cells to certain anticancer drugs in tissue culture, and this approach should be investigated for its potential therapeutic value /60/.

Tumour cell membranes are potentially important targets for selective chemotherapeutic attack /61/. Further research is needed to elucidate the functional consequences of structural and

conformational changes in cell membrane molecules, in order to permit the development of new classes of selectively toxic antitumour drugs (for a review see /62/).

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