reactions seen in the mice are proportional to the doses given, and only detectable when the mice are exposed to very high doses exceeding 40 mg/kg of body weight. Quantitative studies are not possible *in vivo*, when the relationship between the number of particles taken up per cell cannot be satisfactorily controlled. For such studies, it is necessary to use suitable *in vitro* systems, *e.g.*, cultured peritoneal macrophages, where the relationship between the number of particles and cells can be carefully controlled. Such studies will be presented separately, showing when exposure of cultured macrophages to microspheres will lead to changed intracellular function and cellular degeneration. Subsequent *in vivo* applications with acrylic microspheres must be based on such studies.

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Poly-L-methionine Sulfoxide: A Biologically Inert Analogue of Dimethyl Sulfoxide with Solubilizing Potency

JOSEF PITHA *x, LAJOS SZENTE *, and JUDITH GREENBERG ‡

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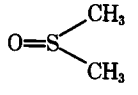
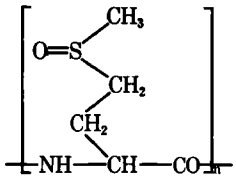
Abstract □ Poly-L-methionine sulfoxide is a water-soluble polymer containing the sulfoxide moiety. The preparation and radiolabeling of this polymer is described and its bioeffects are compared with those of dimethyl sulfoxide. Poly-L-methionine sulfoxide is similar to dimethyl sulfoxide in that it is a potent solubilizer of lipophilic compounds in water. Although the partition coefficient of poly-L-methionine sulfoxide in 1-octanol–water is only 20 times lower than that of dimethyl sulfoxide, it was found not to penetrate into intracellular spaces. In contrast to dimethyl sulfoxide, poly-L-methionine sulfoxide and L-methionine sulfoxide were found to be ineffective in inducing differentiation in murine erythroleukemia cells and inhibiting differentiation of avian neural crest cells, suggesting that compounds effective in these processes must have the ability to penetrate into cells or membrane proteins. Overall lack of bioactivity of poly-L-methionine sulfoxide, combined with low toxicity (2 g/kg, iv, in the mouse with no effect), makes this compound a suitable inert solubilizer and carrier for lipophilic drugs.

Keyphrases □ Dimethyl sulfoxide—poly-L-methionine sulfoxide, biologically inert analogue with solubilizing potency □ Poly-L-methionine sulfoxide—biologically inert analogue of dimethyl sulfoxide with solubilizing potency □ Solubilization potency—poly-L-sulfoxide, biologically inert analogue of dimethyl sulfoxide

A majority of the applications of polymers in pharmacy are based on their mechanical properties and inertness. Also, polymers are able to form complexes with pharmaceuticals; this property has been used relatively rarely, *e.g.*, in decreasing the aggressive properties of iodine by complexation with povidone. Furthermore, polymers possessing complexing power can be used, instead of organic



Dimethyl Sulfoxide



Polymethionine Sulfoxide

solvents, to dissolve nonpolar drugs in aqueous media and to promote sorption of pharmaceuticals. Dimethyl sulfoxide (I), one of the solvents often used for this purpose (1, 2), is relatively nontoxic to cells, yet easily penetrates cellular membranes and, in some cases, triggers very complex biological responses, *e.g.*, induction (3–8) and inhibition (9–12) of cellular differentiation.

It may be advantageous, for both practical applications and for studies of the induction mechanism, to supress some of its bioeffects, *i.e.*, to make a new selective agent by chemical modifications from a pluripotent compound. This may be possible by preparing a macromolecule that contains structural elements of dimethyl sulfoxide. A

Table I—Solubility (μg/ml) of Lipophilic Compounds in Phosphate-Buffered Isotonic Saline in the Presence (5%) or Absence of Poly-L-methionine Sulfoxide

Compound	Saline	Saline and Poly-L-methionine Sulfoxide
β-Ionone, $C_{13}H_{20}O$	7.3	1400
Retinol, $C_{20}H_{30}O$	<4.6	12
β-Carotene, $C_{40}H_{56}$	Nondetectable	6
Lycopene, $C_{40}H_{56}$	Nondetectable	4
Vitamin D_3, $C_{27}H_{44}O$	<0.18	138

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Table II—Distribution of the Studied Compounds between Murine Erythroleukemia Cells and Serum-Free Medium

Compound	cpm Added	cpm in Cell Pellet	cpm Recovered in Pellet and Supernatant, %	cpm in Non-Cellular Component of Pellet[a]	cpm per 10^8 Cells	Compound per Cell, fg[b]
Inulin	50,000	4367	91	4367	0	—
	25,000	2986	96	2986	0	—
	25,000	2520	99	2520	0	—
Poly-L-methionine sulfoxide	50,000	4723	102	5126	(−403)	—
	50,000	4674	102	5126	(−452)	—
	25,000	2668	95	2563	105	—
	25,000	2748	101	2563	185	—
Dimethyl sulfoxide	52,000	7831	99	5331	2,500	1.1
	52,000	7670	103	5331	2,339	1.0
	26,000	4192	106	2666	1,526	0.6
	26,000	4448	106	2666	1,782	0.8

[a] Calculated on the assumption that inulin does not absorb or penetrate cells. [b] Femtograms.

nondegradable polymer containing the structural elements of dimethyl sulfoxide was prepared previously by a multi-step synthesis (13). In contrast to that study, this study describes a facile synthesis of a degradable polymer: poly-L-methionine sulfoxide.

EXPERIMENTAL

Preparation of Poly-L-methionine Sulfoxide—A saturated aqueous solution of sodium periodate (321 mg) was added slowly to a stirred solution of 224 mg of poly-L-methionine[1] (average molecular weight 30,000) in 7 ml of acetic acid at room temperature. Stirring was continued for 12 hr. Water (14 ml) was then added to the reaction mixture, the solution was dialyzed against water, clarified by centrifugation (10,000 rpm for 10 min), and freeze-dried. This procedure yielded 120 mg of colorless polymer. To quantitate the extent of oxidation, the polymer was dissolved in deuterium oxide and its NMR spectrum measured on a 60-MHz spectrometer[2]. Resonance of the methyl group of sulfoxide residues occurs at 2.75 ppm and that of sulfone residues occurs at 3.15 ppm; no signal attributable to methylthio groups could be detected. From the integration of the spectrum it was calculated that the sulfoxide methyl group predominates over the sulfone methyl group by a ratio of 5:1.

Tritiation of Poly-L-methionine Sulfoxide—Poly-L-methionine sulfoxide (4 mg) was added to a solution of 3.5 ml of benzene containing [^{3}H]ethyl methanesulfonate (225 μCi, 0.4 mg)[3]. The mixture was stirred for 15 min, frozen, kept at −20° overnight, and then kept at 80° for 1 week; the solvent was then evaporated. The residue was dissolved in water and dialyzed against water. The residue contained 1 μCi of radioactive polymer (3 mg).

All other compounds used were obtained commercially.

Solubilization of Lipophilic Compounds by Poly-L-methionine Sulfoxide—Excess lipophilic compound (5–10 mg) was enclosed in a test tube (polyethylene, volume 1.2 ml) with phosphate-buffered isotonic saline (1 ml) lacking or containing poly-L-methionine sulfoxide (50 mg). The suspension was stirred by rotating the test tube for 1 day in the dark at 20–22°. Thereafter the suspension was centrifuged and the concentration of lipophilic compound in the clear supernatant was determined spectrophotometrically.

Measurement of Distribution of Compounds Between 1-Octanol and Water—Compounds were equilibrated with the above solvents for 6–24 hr at 20–22° and their concentration determined by liquid scintillation counting (dimethyl sulfoxide) or spectrophotometrically (poly-L-methionine sulfoxide, 210-nm wavelength).

Measurement of Distribution of Compounds Between the Cells in Culture and the Medium—The assay contained a 0.2-ml pellet of murine erythroleukemia cells[4] (14) (1×10^8 cells) and the medium[5] in addition to the radioactive compound studied, in a total volume of 1 ml. The equilibrium distribution of the compound studied was established by incubation for 2 hr at 37°. The cells and medium were separated by centrifugation and the concentration of the compound was calculated from the results of liquid scintillation counting.

Induction and Inhibition of Cell Differentiation—Murine erythroleukemia cells were grown in suspension cultures in medium[5] supplemented with 10% calf serum and antibiotics. The cells were exposed to a potential inducer for 5 days, and their differentiation was assayed by calculating the percentage of cells that were stained by benzidine (14).

Cultures of avian neural crest cells were initiated as described previously (12). Cells were subcultured in replicate plates (10,000–15,000 cells/plate) 2 days after explantation (day 2) and grown in a 1:1 mixture of medium[6] and broth[7] supplemented with 2% chick embryo extract and 5% calf serum.

The assay on neural crest cells was run as follows. Cells were grown in the presence or absence of the test compound from day 2 to 7 of culture. On day 7 fresh medium containing the test compound was added. Cells were then labeled for 5 hr with 5 μCi/ml of [^{14}C]tyrosine[8]. Incorporation of radioactivity into the trichloroacetic acid-insoluble fraction of the cells and melanin-specific incorporation was determined as described previously (12). All values given represent means of duplicates that differed by <10%.

RESULTS

The easiest procedure for preparing poly-L-methionine sulfoxide is the one involving the mild oxidation of poly-L-methionine. Various agents oxidize sulfides to sulfoxides (15, 16). *m*-Chloroperbenzoic acid, iodine, and sodium periodate were tested as oxidizing agents. When sodium periodate was used, no detectable methylthio residues remained, and the overoxidation of the sulfoxide group to the sulfone moiety was only ~16% of the total. Radioactive labeling of poly-L-methionine sulfoxide was performed by an exchange of some of the methyl groups in the polymer for [^{3}H]ethyl groups; since only ~0.05% of these groups were exchanged, the effects of such an introduced structural change on the polymer properties may be disregarded. This labeling occurred using only small amounts of radioactive [^{3}H]ethyl methanesulfonate (0.4%); however, good yields of ^{3}H-polymer (75%) were readily obtained in this manner.

Poly-L-methionine was found to be a potent solubilizer of organic compounds. A series of terpenoid-like lipophilic compounds containing up to 40 carbon atoms was evaluated (Table I).

Next, the partition coefficients (1-octanol–water) of dimethyl sulfoxide and poly-L-methionine sulfoxide were measured. The partition coefficient of dimethyl sulfoxide was found to be 0.038 ($\sigma = 0.007$, $N = 10$). This value compares with 0.01 and 0.04 reported previously (17, 18). The partition coefficient of poly-L-methionine sulfoxide was found to be 0.002 ($\sigma = 5 \times 10^{-5}$, $N = 3$).

The partition of a compound between the cultured cells and surrounding medium is a good indicator of the ability of that compound to be adsorbed by and penetrate cells, the latter process being the important one in equilibrium. For calculation of such distribution from the directly measurable concentrations of the compound in the medium and in the cell pellet, it is necessary to estimate the amount of medium that is retained in the cell pellet. Inulin, an electroneutral polysaccharide which does not penetrate into cells and is only slightly adsorbed by cells, was used for this estimate. The results (Table II) show that poly-L-methionine sulfoxide is excluded from cells to the same extent as inulin. On the other

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Varian Instrument Co., Cheverly, Md.
[3] New England Nuclear, Boston, Mass.
[4] Obtained from the laboratory of Dr. Philip Leder, Bethesda, Md.
[5] Minimum Essential Medium (Eagle, without serum).
[6] Minimum Essential Medium, Delbecco.
[7] Ham's F-12.
[8] New England Nuclear, Boston, Mass.; sp. act. 512 mCi/mmole.

Table III—Induction of Differentiation of Murine Erythroleukemia Cells by Studied Compounds

Compound	Concentration, %	Benzidine-Positive Cells; 5th Day of Induction, %
—	—	1.6
Dimethyl sulfoxide	1	28.0
	1.5	39.0
	2.0	76.0
Poly-L-methionine sulfoxide	0.2	0.2
	0.5	2.5
	1.5	0.0[a]
L-Methionine sulfoxide	1.0	0.0
	1.5	0.0[a]

[a] No cell growth.

hand, 0.6–1.1 fg of dimethyl sulfoxide were taken up per cell. The partition coefficient (concentration in cells/concentration in medium) of dimethyl sulfoxide, when calculated from these measurements, is 0.52 ($\sigma = 0.1, N = 4$).

The ability of the test compounds to induce differentiation of erythroleukemia cells was assessed by calculating the percentage of hemoglobin-synthesizing cells (*i.e.*, benzidine positive) on the 5th day of exposure to the agent. Dimethyl sulfoxide increased the percentage of benzidine-positive cells up to 40 times over that of the untreated controls (Table III). In contrast, poly-L-methionine sulfoxide was completely inactive. For further comparison L-methionine sulfoxide was also included in the test and found inactive (Table III). Concentrations up to the toxic range were tested (Table III), and additional measurements at longer exposure times were also performed to confirm the above conclusions (results not given).

Dimethyl sulfoxide is known to inhibit differentiation of neural crest cells (melanin synthesis) (12). The effects of dimethyl sulfoxide, poly-L-methionine sulfoxide, and L-methionine sulfoxide on the incorporation of [^{14}C]tyrosine into melanin were compared (Table IV). Dimethyl sulfoxide, as expected, inhibited incorporation, whether it was added to the cells only during the labeling period or was present throughout the culture period. In contrast, poly-L-methionine sulfoxide and L-methionine sulfoxide were inactive at 0.05%.

Toxic effects of poly-L-methionine *in vitro* in culture were relatively insignificant. To measure the toxicity of poly-L-methionine *in vivo* doses of 500, 1000, and 2000 mg/kg were injected intravenously into mice (strain C57B1/6J, 9 weeks old, females) using a peristaltic pump system described previously (19). No toxic effects or weight losses were observed when compared with controls, even with the highest dose.

DISCUSSION

Poly-L-methionine sulfoxide, in contrast to poly-L-methionine, is very water soluble. This solubility, its one-step preparation, and its ease of radiolabeling, make it suitable for biological studies.

Preparation of a macromolecular analogue of a pharmacon often leads to the elimination of some of its effects. Of the various bioeffects of dimethyl sulfoxide, conversion to the macromolecular poly-L-methionine sulfoxide, resulted in the retention of only the solubilization effects. Compounds used in solubilization tests (Table I) are structurally related. β-Ionone, a monocyclic terpene with a short side chain, was efficiently solubilized, whereas retinol, which contains the same monocyclic structure but has a longer side chain, was only moderately solubilized. β-Carotene and lycopene were solubilized to a moderate extent. These data suggest that poly-L-methionine sulfoxide preferentially solubilizes compounds which have a compact structure, *i.e.*, without long hydrocarbon chains. Vitamin D_3, which has such a compact structure, was indeed efficiently solubilized.

Partition coefficients of drugs and other organic compounds between 1-octanol and water have been used extensively in the correlation of their bioeffects. This coefficient is a measure of the ability of the drugs to interact and penetrate the lipid bilayer of cellular membranes, which is thought to be well approximated by 1-octanol. Using this established comparison system it was found that poly-L-methionine sulfoxide distributes less uniformly (0.002) than dimethyl sulfoxide (0.04). The value of the distribution coefficient of poly-L-methionine sulfoxide *per se* is not low enough to lead to the assumption that no penetration into cells or bioeffects may be achieved by this compound (18). Probably, the macromolecular character of poly-L-methionine sulfoxide is another

Table IV—Effect of Compounds on Pigmentation of Neural Crest Cells

Compound	Concentration, %	[^{14}C]Tyrosine Incorporation into Melanin, cpm/cell	
		Day 7	Days 2–7
—	—	0.008	0.004
Dimethyl sulfoxide	1	0	0
Poly-L-methionine sulfoxide	0.5	Not determined	Toxic
	0.05	0.007	0.006
L-Methionine sulfoxide	0.05	Not determined	0.006

contributing factor leading to the observed inertness. Poly-L-methionine sulfoxide was found, as described in the *Results* section, not to penetrate into the cellular spaces and, thus, may only interact with the external surface of cellular membranes.

In the context of the study on the mechanism of changes in cell differentiation induced by dimethyl sulfoxide it is important to note that its nonpenetrating analogue, poly-L-methionine sulfoxide, failed to affect cell differentiation in either of the systems tested. These findings suggest that the mechanism of action of dimethyl sulfoxide requires its entry into cells or into the hydrophobic interior of some protein on the cell surface, a process that can change the structure and function of the protein. The lack of penetration of poly-L-methionine sulfoxide into cells was proven experimentally; entry of a compound into the hydrophobic sections of proteins is possible only for small molecular weight hydrophobic or amphophilic molecules and, thus, poly-L-methionine sulfoxide is automatically excluded (20). A low molecular weight, highly polar analogue of dimethyl sulfoxide, L-methionine sulfoxide, also lacked any inducing ability (Tables III and IV).

In addition to the lack of cell-inducing activity, poly-L-methionine sulfoxide has low acute toxicity, even when given intravenously.

Thus, the most promising application of poly-L-methionine sulfoxide seems to be in the complexing of bioactive lipophilic compounds. This complexing converts the lipophilic compounds into water soluble ones; thus, to an extent poly-L-methionine sulfoxide may functionally imitate carrier serum proteins. Such complexing of drugs has often led to a decrease in their toxicity, as shown, for example, in the case of retinoids (21). Complexing of drugs with poly-L-methionine may possibly have the same consequences.

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Nonisothermal Kinetics Using a Microcomputer: A Derivative Approach to the Prediction of the Stability of Penicillin Formulations

J. M. HEMPENSTALL *, W. J. IRWIN *x, A. LI WAN PO*, and A. H. ANDREWS ‡

Received March 2, 1982, from the **Department of Pharmacy, University of Aston in Birmingham, Gosta Green, Birmingham, B4 7ET, UK,* and the ‡ *Beecham Pharmaceuticals, Research Division, Worthing, West Sussex, BN14 8QH, UK.* Accepted for publication June 9, 1982.

Abstract □ A procedure is described for the determination of the shelf-life of pharmaceutical preparations using nonisothermal kinetics. A BASIC computer program, which enables the data analysis to be undertaken rapidly and automatically on a microcomputer, is presented.

Keyphrases □ Kinetics, nonisothermal—derivative approach to the prediction of the stability of penicillin formulations using a microcomputer □ Stability—nonisothermal kinetics using a microcomputer, a derivative approach to the prediction, penicillin formulations □ Penicillin—nonisothermal kinetics using a microcomputer, a derivative approach to the prediction of stability in formulations □ Formulations—penicillin, nonisothermal kinetics using a microcomputer, a derivative approach to the prediction of stability

Nonisothermal methods for the prediction of the shelf-life of pharmaceutical preparations are an attractive alternative to traditional isothermal accelerated storage tests. A nonisothermal study involves a temperature change throughout the reaction and enables a full stability–temperature profile to be determined from one experiment. This procedure offers a considerable reduction in effort for the estimation of shelf-life and has received much attention. Early methods (1, 2) were extended to pharmaceuticals by Rogers (3) who used a defined temperature rise profile to simplify data handling. The validity of this approach, using various heating programs, has been confirmed (4–8), but theoretical and practical limitations have been discussed (9, 10). Greater freedom in experimental design is available if a predetermined temperature–time profile is not demanded. Methodology has therefore been extended to allow the rate of temperature increase to be determined by experimental, rather than theoretical, expedience (11, 12). Nonisothermal–isothermal methods have also been reported (13, 14), and many important applications of nonisothermal kinetics to solid-state degradations have appeared (15, 16). The estimation of the errors involved in these procedures has also received attention (12, 17).

Despite the success of these methods, few applications to formulated products have appeared (18). In this study a general method for the determination of nonisothermal kinetic profiles is described, and a BASIC computer program (NONISO) is presented, which can be implemented on a microcomputer, to undertake the calculations automatically. The procedures are shown to be comparable to methods requiring large computing facilities (12) and prove satisfactory for formulated products.

THEORETICAL

Degradation Rates—The rate of degradation of a drug can be represented by:

$$\frac{-dC_t}{dt} = k_T C_t^n \qquad \text{(Eq. 1)}$$

where C_t is the concentration at time t, k_T is the specific rate constant at temperature T, and n is the order of reaction. For a first order reaction ($n = 1$) this can be written as:

$$C_t = C_0 e^{-k_T t} \qquad \text{(Eq. 2)}$$

In logarithmic form this is:

$$\ln C_t = \ln C_0 - k_T t \qquad \text{(Eq. 3)}$$

and the negative slope of the plot k *versus* $\ln C_t$ yields the rate constant.

When the temperature is continually increased throughout the reaction, the degradation rate progressively increases. The isothermal rate constant is now approximated by:

$$k_T = -\left[\frac{\ln C_t - ln C_{t-\delta t}}{\delta t}\right] \qquad \text{(Eq. 4)}$$

where δt is a small increment of time, over which period the temperature may be considered constant. For an infinitesimal increase in time and temperature, the specific rate constant is given by:

$$k_T = -\frac{d(\ln C_t)}{dt} \qquad \text{(Eq. 5)}$$

The slope of the tangent at a point for the plot of t *versus* $\ln C_t$ for nonisothermal data yields the specific rate constant at the temperature observed.

If other orders of reaction are followed the appropriate equations are:

$$\text{zero order:} \quad k_T = -d(C_t)/dt \qquad \text{(Eq. 6)}$$

$$\text{2nd order (a = b):} \quad k_T = d(1/C_t)/dt \qquad \text{(Eq. 7)}$$

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