Dispos., 4, 577 (1976).
(14) S. Nicolaidis, N. Rowland, M. Meile, P. Marfaing-Jallat, and A. Pesez, *Pharmacol. Biochem. Behav.*, 2, 131 (1974).
(15) S. Y. Chang, D. S. Alberts, D. Farquhar, L. R. Melnick, P. D. Walson, and S. E. Salmon, *J. Pharm. Sci.*, 67, 682 (1978).

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Hydrolysis and Protein Binding of Melphalan

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Abstract □ Melphalan (30 μg/ml) is completely hydrolyzed in water at 37° after 8 hr. At lower temperatures, hydrolysis proceeds at slower rates. The presence of bovine serum albumin retards hydrolysis of melphalan (30 μg/ml) in water. The melphalan hydrolysis rate is directly related to the bovine serum albumin concentration. At 37°, 8 g of bovine serum albumin/100 ml of water gives a recovery rate of melphalan similar to that of human plasma. *In vitro* alkylation of melphalan at 37° with human plasma containing 30 μg/ml, calculated by equilibrium dialysis, methanol extraction, and high-pressure liquid chromatographic analysis, is 30% after 8 hr.

Keyphrases □ Melphalan—hydrolysis, effect of bovine serum albumin, various temperatures □ Hydrolysis—melphalan, effect of bovine serum albumin, various temperatures □ Antineoplastic agents—melphalan, hydrolysis, effect of bovine serum albumin, various temperatures

The alkylation of nucleic acids and proteins by melphalan (I) has been studied using spectrophotometry, radiochemistry, and equilibrium dialysis (1, 2–4). Chirigos and Mead (2), using fluorometric detection and equilibrium dialysis, showed that 60% of I was covalently bound to plasma proteins at 26°. Other studies (1, 3, 4) also suggested covalent binding of I to proteins and nucleic acids.

It is necessary to understand the *in vitro* hydrolysis and alkylation of I to describe its pharmacokinetics in humans. This report documents the qualitative and quantitative aspects of adsorption, hydrolysis, and alkylation of I.

EXPERIMENTAL

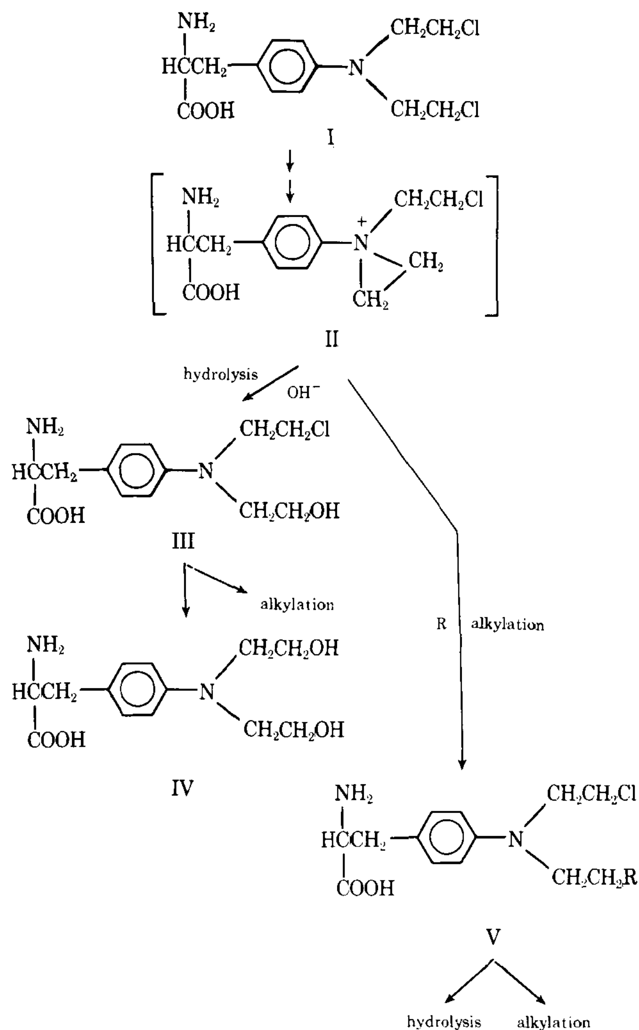
Materials—^{14}C-Melphalan (^{14}C-ethyl labeled) was synthesized according to the method of Bergel and Stock (5) and purified by high-pressure liquid chromatography (HPLC) (6). Dansylproline[1] was used as an internal standard for HPLC.

Assay of I—Compound I was extracted and analyzed using a previously described HPLC method (6).

Recovery Studies—The hydrolysis rate of I to III and IV in water (Scheme I) at 0, 23, and 37° was studied. Thirty-five micrograms of I and 70 μg of internal standard were added to 1 ml of water, and samples were taken at various times for HPLC analysis as previously described (6). The rate constants, k, were calculated according to $k = 2.3 \log ([I]_0/[I]_t)/t$, where $[I]_0$ and $[I]_t$ are the concentrations of I at times zero and t.

To study the effect of protein on the I hydrolysis rate in water, 30 μg of I and 70 μg of internal standard were added to increasing concentrations of bovine serum albumin[2] in water (*e.g.*, 8, 13, and 25 g/100 ml at 37°). At designated intervals, samples were taken for HPLC analysis.

The extractability of I from, and degradation in, human plasma was studied *in vitro* at 37°. Three hundred micrograms of I (1.2 μCi of carbon-14) and 700 μg of internal standard were added to 10 ml of human plasma, and 1-ml aliquots were extracted with methanol at given intervals



Scheme I

[1] Pierce Chemical Co., Rockford, Ill.

[2] Sigma Chemical Co., St. Louis, Mo.

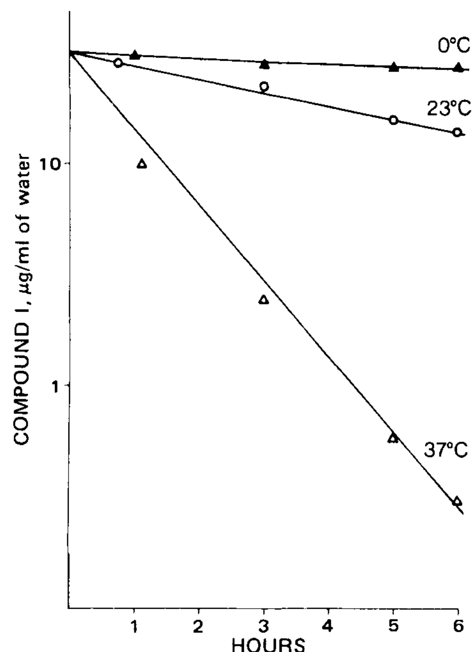


Figure 1—*Recovery of I from water. Compound I was added to distilled water (35 μg/ml) and incubated at 0° (▲), 23° (○), and 37° (△). Samples were taken at designated times and analyzed by HPLC.*

(6). The supernate of the methanol extracts, 0.1 ml, was assayed for total ^{14}C-content. These same samples were separated by HPLC into a 5-ml fraction of I and a 5-ml fraction of III plus IV. Each fraction was mixed with 20 ml of scintillation fluid[3] and assayed for carbon-14. The quenching effects of the HPLC solvent were corrected. At the same time, HPLC analysis of I was carried out, but quantification of III or IV by HPLC was not possible because of plasma background interference.

Equilibrium Dialysis—The dialysis apparatus[4] had a 2-ml capacity. A cellulose acetate membrane separated the system into symmetrical 1-ml chambers. One chamber contained ^{14}C-I (0.1 μCi of carbon-14) and 30 μg of I in 1 ml of human plasma, and the opposite chamber contained 1 ml of 0.05 *M* phosphate buffer (pH 7.4). After gentle shaking, 20 μl was removed from each chamber at intervals up to 25 hr and analyzed for ^{14}C-content. The dialyses were carried out at 4, 14, 23, and 37°.

Additional 4° experiments were performed and analyzed by HPLC. For these experiments, 100 μg of I was added to 1 ml of plasma. The internal standard was not used because of its high adsorption by the dialysis membrane. Compound I concentration was calculated using a standard response curve. Control dialysis experiments were also performed at 4, 14, and 37° in buffer.

RESULTS

Recovery—The hydrolysis of I in water as a function of temperature and incubation time is shown in Fig. 1. A rapid first-order kinetic degradation is shown with rate constants of 0.83/hr (37°), 0.13/hr (23°), and 0.04/hr (0°). The hydrolysis of I in water solutions of bovine serum albumin at 37° as a function of time and bovine serum albumin concentration is shown in Fig. 2. Hydrolysis rate constants of I in bovine serum albumin solution are 0.46/hr (8 g of bovine serum albumin/100 ml), 0.36/hr (13 g of bovine serum albumin/100 ml), and 0.24/hr (25 g of bovine serum albumin/100 ml). The *in vitro* hydrolysis rate constant of I from human plasma varied between 0.31 and ~0.45/hr at 37°.

Extraction of ^{14}C-I—Figure 3 shows the extractability of total carbon-14 from human plasma, the recovery rate of I and ^{14}C-I, and the hydrolysis of ^{14}C-I to ^{14}C-III and ^{14}C-IV at 37°. The recovery of total methanol-extractable carbon-14 at 37° slowly decreased to 70% of the initial concentration after 8 hr (Fig. 3). At any given time point, the sum of ^{14}C-I, ^{14}C-III, and ^{14}C-IV of the HPLC eluates was equivalent to the total methanol extraction of carbon-14 (Fig. 3).

The 75% recovery of ^{14}C-I (Fig. 3) at zero time was due to partial hydrolysis of ^{14}C-I to ^{14}C-III and ^{14}C-IV. The decrease in ^{14}C-I recovery can be accounted for by the increase in ^{14}C-III and ^{14}C-IV.

Dialysis Studies—The results of dialysis experiments at 4, 14, 23, and 37° are shown in Fig. 4. Equilibrium was reached in 8 hr at all temperatures. No adsorption of I to dialysis membranes was observed. At equilibrium, 33% of I remained bound to plasma proteins at 37°. At 23°, undialyzable I decreased to 15–20%. At 4°, 30% of I remained bound to plasma; however, this 30% was extractable with methanol (Table I).

***In Vitro* and *In Vivo* Studies**—Figure 5 shows the *in vitro* recovery of I (12 μg/ml) in plasma at 37° and an *in vivo* plasma decay curve for I (0.6-mg/kg iv bolus injection) in a patient with ovarian carcinoma.

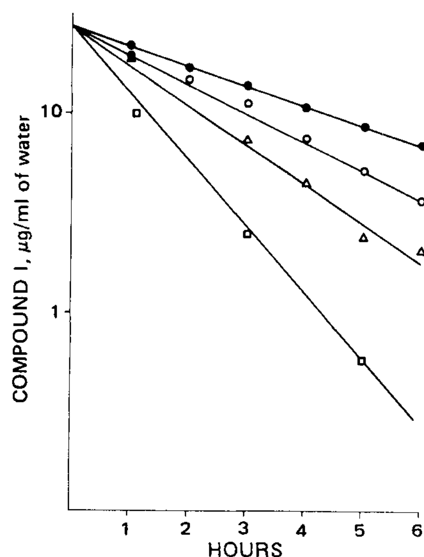


Figure 2—*Effect of bovine serum albumin on the recovery of I. Compound I (30 μg/ml) was added to various concentrations of bovine serum albumin and incubated at 37°. Samples were taken at designated time points and analyzed by HPLC. Key (concentration of bovine serum albumin): □, 0; △, 8 g/100 ml; ○, 13 g/100 ml; and ●, 25 g/100 ml.*

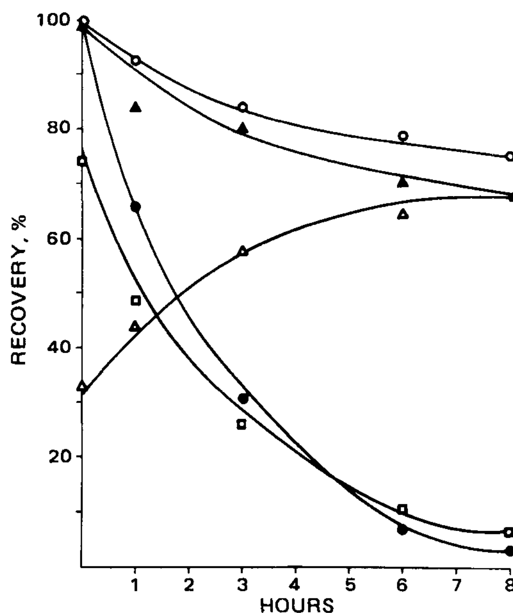


Figure 3—*Methanol extraction of ^{14}C-I and HPLC analyses. ^{14}C-Compound I (1.2 μCi/300 μg of I) was added to human plasma and incubated at 37°. At various times, aliquots were taken and extracted with methanol from which a portion was directly assayed for carbon-14 (▲) and a portion was assayed by HPLC for I (●). The eluate from the chromatograph was assayed for ^{14}C-I (□) and the mixture of ^{14}C-III and ^{14}C-IV (△); ○ denotes the sum total of ^{14}C-I, ^{14}C-III, and ^{14}C-IV of the HPLC eluate.*

[3] Aquasol, New England Nuclear, Boston, Mass.
[4] Chemical Rubber Co., Cleveland, Ohio.

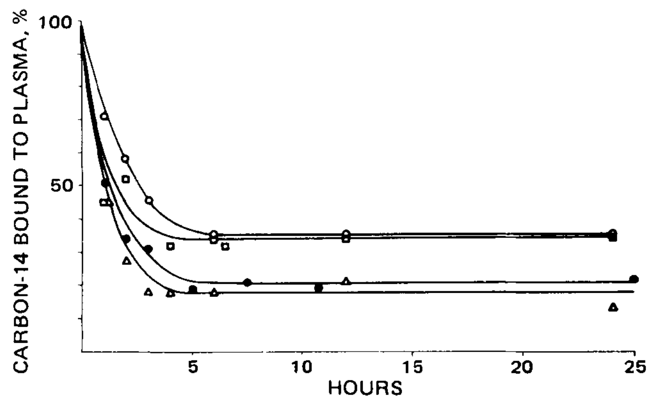


Figure 4—*Equilibrium dialysis of ^{14}C-I. ^{14}C-Compound I (0.1 µCi) and 30 µg of I were added to 1 ml of human plasma and dialyzed with 1 ml of a phosphate buffer (0.05* M, *pH 7.4) at 4° (○), 14° (●), 23° (△), and 37° (□). Samples from both chambers were taken simultaneously at designated times and assayed for carbon-14.*

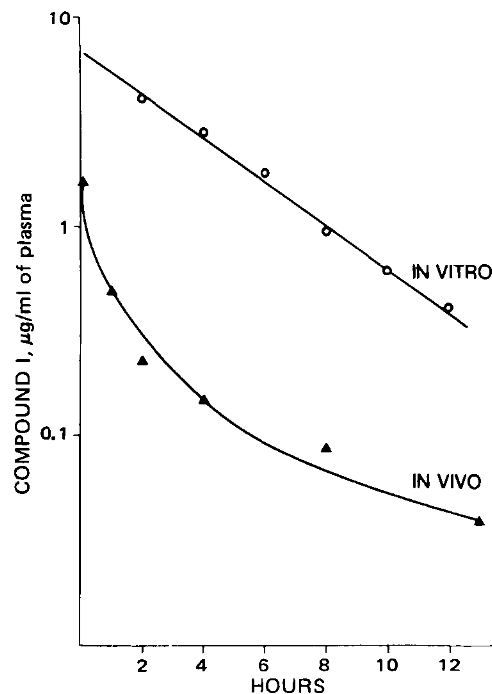


Figure 5—In vitro *and* in vivo *recovery of I from human plasma. Key:* ○, in vitro *recovery of I (12 µg/ml) at 37°; and* ▲, in vivo *recovery after patient received 0.6-mg/kg iv bolus injection.*

DISCUSSION

The hydrolysis of I to III and IV has been described (2, 6, 7). Compound I may form a reactive ammonium ion (II) and then be hydroxylated to III and IV (7–10). In such reactions, the hydrolysis of I would be expected to follow first-order kinetics (10–12). As shown in Figs. 1, 2, and 5, *in vitro* recoveries of I from water, bovine serum albumin, and human plasma followed first-order kinetics.

The present results show that bovine serum albumin or human plasma proteins retarded the hydrolysis rate of I *in vitro*. Linford (13) made a similar observation for chlorambucil. Because the quantification of III and IV by HPLC was not performed due to background interference, the chemical nature of unrecovered I remained undetermined. There is evidence that I undergoes extensive alkylation and physical adsorption to plasma proteins (1–4). Thirty percent of the initial carbon-14 was not extracted from plasma proteins at 37° after 8 hr (Fig. 3). This fact and the results of the dialysis experiments whereby 30% of carbon-14 remained undialyzable at 37° after 8 hr may indicate alkylation of I to plasma proteins.

Chirigos and Mead (2) terminated their dialysis experiments after 3 hr. The present results show that the equilibrium points at 4, 14, 25, and 37° were not reached until 8 hr (Fig. 4). The 30% undialyzable I seen at 4° may have been due to increased adsorption of I to plasma proteins at low temperature. As the temperature was increased, this physical adsorption was reversed (see 14 and 23° in Fig. 4). Also 30% undialyzable I at 4° was extractable with methanol (Table I).

There was a 10% difference at 8 hr between total methanol-extractable carbon-14 and the sum total ^{14}C-eluate of HPLC at times less than 8 hr because of an overcorrection of the quenching effect of the solvent.

The marked difference between the *in vitro* and *in vivo* recovery of I from human plasma is shown in Fig. 5. Clearly, pharmacokinetic studies of I will require careful analysis of spontaneous hydrolysis and protein adsorption as well as of physiological disposition and metabolism. The evidence for significant hydrolysis of I at room temperature in protein-free medium suggests that a parenteral solution of the drug should be prepared immediately prior to administration and should be kept cold or prepared with albumin in the diluent.

Table I—Equilibrium Dialysis of I in Human Plasma at 4° by HPLC Analysis

Time Elapsed	I, µg/ml
Zero time	100.0
1 hr, 10 min	79.9
4 hr, 15 min	52.9
8 hr, 50 min	39.6
21 hr, 30 min	35.0
22 hr	33.5

REFERENCES

(1) P. Cohn, *Br. J. Cancer*, **1**, 258 (1957).
(2) M. Chirigos and J. Mead, *Anal. Biochem.*, **7**, 259 (1964).
(3) A. Milner, O. Klatt, S. Young, and J. Stephlin, Jr., *Cancer Res.*, **25**, 259 (1965).
(4) R. P. Sanderson, J. M. McKenna, and W. S. Blackemore, *Nature*, **205**, 479 (1965).
(5) F. Bergel and J. Stock, *J. Chem. Soc.*, **76**, 2409 (1954).
(6) S. Y. Chang, D. S. Alberts, L. R. Melnick, P. D. Walson, and S. E. Salmon, *J. Pharm. Sci.*, **67**, 679 (1978).
(7) W. C. J. Ross, "Biological Alkylating Agents," Butterworths, London, England, 1962, pp. 19, 32, 60, 99.
(8) "Review of Medical Pharmacology," 5th ed., F. Meyers, E. Jawetz, and A. Goldfien, Eds., Lange Medical Publications, Los Altos, Calif., 1976, p. 470.
(9) R. B. Livingston and S. Carter, "Single Agents in Cancer Chemotherapy," Plenum, New York, N.Y., 1970, p. 100.
(10) W. C. J. Ross, in "Antineoplastic and Immunosuppressive Agents—I," A. Sartorelli and D. Johns, Eds., Springer-Verlag, New York, N.Y., 1974, p. 40.
(11) W. C. Gardiner, Jr., "Rates and Mechanisms of Chemical Reactions," Benjamin, New York, N.Y., 1969.
(12) E. S. Gould, "Mechanism and Structure in Organic Chemistry," Holt, Rinehart and Winston, New York, N.Y., 1959, pp. 159–192.
(13) J. H. Linford, *Biochem. Pharmacol.*, **8**, 343 (1961).

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