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Original Articles

Comparative Physico-chemical Properties, Biological Effects, and Disposition in Mice of Four Nitrogen Mustards

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Summary. *Chlorambucil, phenyl acetic mustard, melphalan and mitoclomine are aromatic nitrogen mustard derivatives. These drugs show a fairly wide range of chemical reactivity and lipophilicity. In the series presently investigated, the more hydrophilic compounds (melphalan and phenyl acetic mustard) are the more toxic to mice and also the more active against Moloney sarcoma implanted IP in mice. No clear relation could be shown between the alkylating activity and the biological efficiency. All the compounds induce changes in DNA synthesis with differences between the timing of alterations and recovery. Nevertheless, return to pretreatment levels of thymidine incorporation is more rapid in the bone marrow and intestinal mucosa following administration of the drugs, than in tumor cells. The ^{14}C-ethyl labeled compounds was used to investigate the part of the carrying structure in their disposition in mice.*

Introduction

Chlorambucil and phenylacetic mustard[1] belong to a group of aniline mustard derivatives synthesized by Everett [11]. The introduction of a carboxylic function was designed to make the parent molecule more hydrophilic. Chlorambucil is widely used in the treatment of chronic lymphoid leukemia (CLL), malignant types of lymphoma and disorders of immune origin such as rheumatoid arthritis and lupus disease [9, 15]. Moreover, it has been recognized to have some specificity for lymphoid cell lines [20]. Melphalan, whose carrying structure is an amino acid-L-phenylalanine, was prepared by Bergel and Stock [3] in the hope that it would allow carrier-mediated transport. It has a wide spectrum of activity and when administered alone or in association with other drugs it is particularly effective in treating myeloma, breast cancer and ovarian cancer [26]. Granger[2] originally synthesized mitoclomine, because the structure carrying the *N,N*-di-(2-chloroethyl) group – synthetic vitamin K_5 – was found to concentrate selectively in certain types of tumoral tissue and to have radiosensitizing properties [25]. Mitoclomine, an alkylating agent which came into use more recently and entered clinical trial phases I–II in 1975, has exhibited significant activity in patients suffering from CLL [21].

For several years, our laboratory has been interested in the disposition and metabolism of these drugs in animals. Although chlorambucil and melphalan have now been used in treating human subjects for 20 years, little information had been published in the literature about their metabolism. Phenylacetic mustard was identified as the main blood metabolite of chlorambucil in animals [16, 22] and more recently in man [1, 23, 29]. Furthermore, Mitoma et al. [27] have characterized ten metabolites of chlorambucil in urine of rats. Most of them were mustards or half-mustards of phenylacetic or benzoic acid derivatives. Mitoclomine was shown to undergo a dealkylation reaction leading to formation of an hydroxylated mustard in the para position of the alkylation function [17]. Connors et al. [8] stressed the high toxicity of this group of nitrogen mustards. The metabolism of melphalan is still insufficiently known, but Furner et al. [14] are reportedly on the point of identifying its chief metabolites in the dog and Chang et al. in man [5].

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1 Abbreviation for *N,N*-di-(2-chloroethyl)-4-amino phenylacetic acid

2 Granger, CD (1967) Proceedings of International Congress of Chemotherapy, Vienna

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The present study was undertaken, concomitantly with our current metabolic investigation, to compare and measure under identical well-codified experimental conditions the chemical and physicochemical properties of these four nitrogen mustards, some of their biological effects, and their disposition in animals.

Materials and Methods

Drugs

Chlorambucil, phenylacetic mustard, melphalan and mitoclomine (for formulae, see Table 1) were synthesized and labeled with ^{14}C on the dichloroethyl function at INSERM Unit 71, Clermont-Ferrand, France [28, 30, 24]. Chemical purity was checked by thin-layer chromatography (t.l.c.), i.r., n.m.r., and mass spectrometry. The radiochemical purity of the ^{14}C-labeled compounds, as determined by t.l.c., was > 97%. Specific radioactivities were as follows: ^{14}C-chlorambucil, 3.2 μCi/mg; ^{14}C-phenylacetic mustard, 0.3 μCi/mg; ^{14}C-melphalan, 4.8 μCi/mg; ^{14}C-mitoclomine, 3.5 μCi/mg.

In vitro Studies

Hydrolysis Rate. The hydrolysis rate was measured as defined by Ross [34], i.e., as the relative hydrolysis rate of each drug in a 50% aqueous-acetone solution for 30 min at 66° C, at a final concentration of 0.02 *M*. The products formed (unchanged molecule, mono and dihydroxyl derivatives) were analyzed by t.l.c. (plastic sheets precoated with silica gel, Merck, Darmstadt) in the following elution systems: chlorambucil and phenylacetic mustard (ethylacetate-cyclohexane 70 : 30 v/v); melphalan (*n*-butanol-acetic acid-water 40 : 10 : 10 v/v); mitoclomine (ethylacetate-cyclohexane 30 : 70 v/v). Chromatograms were then cut into strips 0.5 cm wide and their radioactivity was measured in a Mark II Nuclear Chicago liquid scintillation spectrometer.

Alkylating Activity. To 0.2 ml unlabeled alkylating agent dissolved in a 5 : 5 acetone-ethanol mixture were added 1 ml 0.025 *M* sodium acetate buffer, pH 6, and 1 ml 5% paranitrobenzyl pyridine (NBP) in acetone. After a 2-h incubation at 37° C, 3 ml 25% 3-amino-1-propanol in tertiary butanol was added, and the coloration was measured at 560 nm in a spectrophotometer [7].

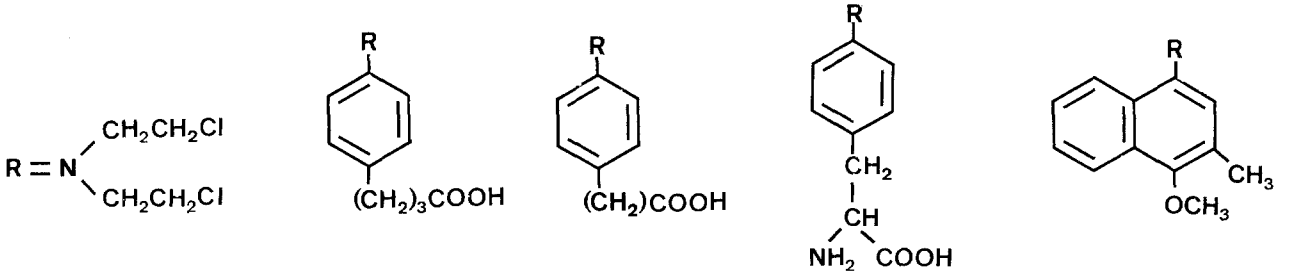
Partition Coefficients. At a final concentration of 0.02 *M*, the ^{14}C-labeled compounds were added to 2 ml benzene and 2 ml phosphate buffer, pH 7.4. After vigorous shaking, the two phases were separated by centrifugation and the radioactivity of each phase was measured. Chromatographic verification of the organic and aqueous phases by t.l.c. showed no significant hydrolysis during the partition process.

In vivo Studies

For all experiments in animals, compounds were dissolved in a mixture of 5% ethanol and 95% oil just before injection.

Toxicity. LD_{50} values were determined by the Spearman-Karber method [12].

Table 1. Structures, physicochemical parameters, and biological activity of the drugs



	Chlorambucil	Ac-Chlorambucil	Melphalan	Mitoclomine
Total hydrolysis rate	21.5%	15.5%	17.0%	60%
Monohydroxyl	17.1%	12.5%	12.5%	14%
Dihydroxyl	4.4%	3.0%	4.5%	46%
Alkylating activity[a]	100	119	66	370
Partition coefficient[b]	0.67	−0.05	−1.70	2.8
LD 50 (mg/kg)	38	21	23	240
Percent ILS[c]	146	182	170	67

[a] Percent of chlorambucil

[b] Determined in benzene/water. Expressed as log P

[c] 2×10^6 Moloney's sarcoma cells were implanted IP at day 0. At day 5 of tumor growth, groups of 10 tumor-bearing mice were given each of the four drugs (half LD_{50}) IP; the control group of 30 mice was only given the solvent

$$\text{Percent ILS} = \frac{\text{Mean survival time of treated mice}}{\text{Mean survival time of control mice}} \times 100 - 100$$

Survival Time. Female C57 BL6 mice were injected IP with 2×10^6 cells (Moloney's sarcoma in ascitic form, MBL_2 line). On day 5 of tumor growth, a single dose of the four nitrogen mustards was injected IP. Doses corresponded to each drug's half-LD_{50}: chlorambucil, 19 mg/kg; phenylacetic mustard, 10 mg/kg; melphalan, 12 mg/kg; mitoclomine, 120 mg/kg. Control groups were only given the solvent. Survival time was recorded for each treated group and compared with the controls' survival.

In vivo DNA Synthesis

Drug effects on DNA synthesis were determined in mice injected IP with 2×10^6 Moloney's sarcoma cells. On day 5 of tumor growth, groups of three mice were given 10 μCi ^{3}H-thymidine IP (CEA Saclay, France; sp. act. 5 Ci/mmole), decapitated 1 h after injection, and used as controls. Groups of mice were treated IP with each drug (half LD_{50}). Between 0.5 h and 168 h after drug administration, animals received 10 μCi ^{3}H-thymidine IP and 1 h later they were sacrificed. Ascites were aspirated and the peritoneal cavity was rinsed with 2×3 ml MEM (minimum Eagle's medium). The thymus and spleen were removed, and the intestinal mucosa was scraped off the inner wall of the duodenum. The bone marrow was harvested from both tibias. Samples from three mice were pooled. DNA was extracted by a minor modification of Schmidt-Tannhauser's technique [36, 39]. An aliquot of the final supernatant containing the DNA was used for radioactivity measurement and another aliquot for DNA determination by Burton's diphenylamine method [4].

Disposition in Mice. Twenty-gram C57 BL6 mice were given equimolecular doses of the ^{14}C-labeled compounds (80 μmol/kg) IP. Animals were sacrificed by ether inhalation at different intervals after injection. Blood was drawn by cardiac puncture and tissue samples were taken from the liver, kidneys, spleen, lymph nodes, thymus, and muscle. Urine and feces were collected separately. Total radioactivity was measured after combustion in an IN 4101 Intertechnique sample oxidizer. Whole-body mouse autoradiographies were carried out according to the technique described by Ullberg [37].

Results

Physicochemical Properties

Table 1 gives the drugs' structures and their chemical and physicochemical parameters measured in vitro. There was little difference between hydrolysis rates for chlorambucil, phenylacetic mustard, and melphalan, which were low compared with the rate for mitoclomine. Alkylating activities for chlorambucil and phenylacetic mustard were not significantly different, whereas the activity for melphalan was definitely lower. Mitoclomine displayed excellent reactivity in relation to the nucleophilic agent used. The greatest disparity among the four molecules was in their partition coefficients: thus, mitoclomine proved strongly lipophilic in relation to melphalan, which dissolved easily in water; chlorambucil and phenylacetic mustard occupied an intermediate position, the latter drug being distributed in equal proportions between the organic and aqueous phases.

Biological Effects

Toxicity. Comparison of LD_{50} showed chlorambucil to be less toxic than its metabolite phenylacetic mustard, whose toxicity was close to that of melphalan. Mitoclomine was by far the least toxic of these drugs (Table 1).

Survival Time. The percentage increases in life-span (percent ILS) of mice bearing Moloney's sarcoma are shown in Table 1. Phenylacetic mustard and melphalan seemed the most effective agents against Moloney's sarcoma, the average survival time after injection of these drugs being 17.8 and 17.0 days, respectively, from the start of treatment. Chlorambucil, and more especially mitoclomine, were less effective in increasing survival (15.2 and 10.5 days, respectively). Mean survival time for the control group was 6.3 days (Fig. 1).

Drug Effects on DNA Synthesis. The curves in Fig.2 show the changes the four drugs induced in DNA synthesis in different types of normal mouse tissue. The inhibitory effects of chlorambucil and phenylacetic mustard were the first to appear: by 6 h after treatment the different tissues examined all showed a

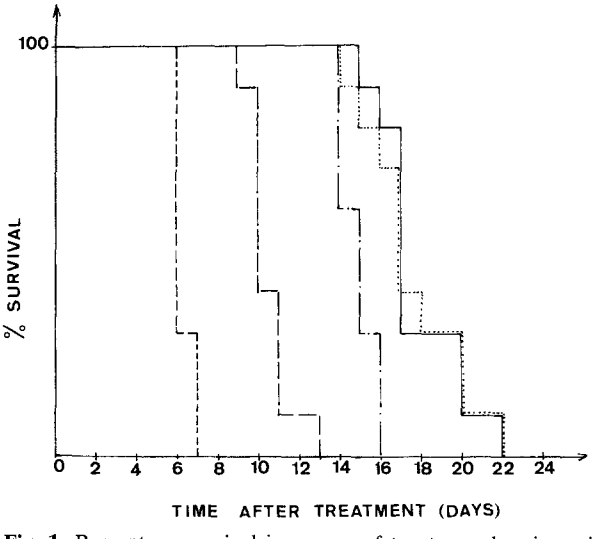


Fig. 1. Percentage survival in groups of ten tumor-bearing mice (Moloney sarcoma in ascitic form) given IP at day 5 of tumor growth: chlorambucil 19 mg/kg (— · —); Ac-chlorambucil 10 mg/kg (———); melphalan 12 mg/kg (· · · · ·); mitoclomine 120 mg/kg (— — —); control (- - - - -)

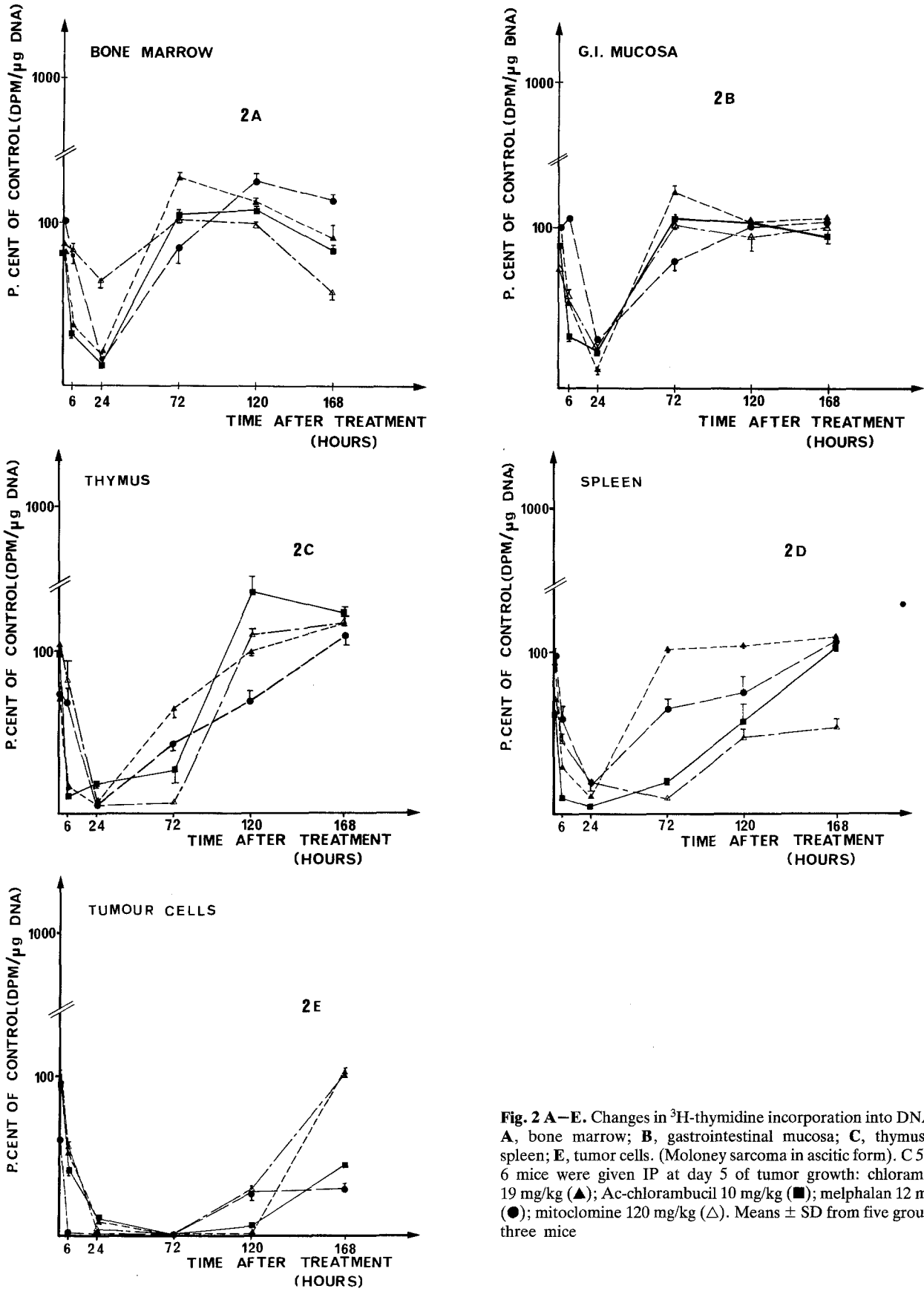


Fig. 2 A–E. Changes in ^{3}H-thymidine incorporation into DNA of: **A**, bone marrow; **B**, gastrointestinal mucosa; **C**, thymus; **D**, spleen; **E**, tumor cells. (Moloney sarcoma in ascitic form). C 57 BL 6 mice were given IP at day 5 of tumor growth: chlorambucil 19 mg/kg (▲); Ac-chlorambucil 10 mg/kg (■); melphalan 12 mg/kg (●); mitoclomine 120 mg/kg (△). Means ± SD from five groups of three mice

sharp drop in their respective precursor incorporation rates, which fell to less than half the control rates. For all drugs, precursor incorporation was at its nadir at 24 h. Recovery times were shorter for bone marrow and intestinal mucosa than for the thymus and spleen. Mitoclomine was less effective in reducing DNA synthesis in bone marrow but the spleen proved especially sensitive to this drug. Tumor cells virtually stopped incorporating ^{3}H-thymidine between 24 h and 120 h (E in Fig. 2). At 168 h after treatment mouse ascites injected with either chlorambucil or mitoclomine displayed a normal incorporation level. At the same time point there was still a definite deficiency in DNA synthesis in the tumor cells of mice injected with melphalan and phenylacetic mustard.

Disposition in the Mouse

Table 2 gives the concentrations expressed as picomoles of drug equivalent per milligram of tissue for the four ^{14}C-ethyl compounds in the different types of tissue. Appearance of the label in blood is more rapid in mice given ^{14}C-chlorambucil and ^{14}C-phenylacetic mustard than in mice injected with ^{14}C-melphalan or ^{14}C-mitoclomine. Peak blood radiactivity occured at 0.5 h and showed a high absorption rate for the labeled species of both chlorambucil and phenylacetic mustard. The evolution of blood radioactivity as a function of time, after injection of ^{14}C-melphalan, was distinctly different, since the highest concentration was not reached until 12 h. After ^{14}C-mitoclomine administration blood concentration of the label remained very low from 0.5 h to 48 h. Radioactivity was widely distributed in the liver and the kidney following all four drugs. There were, however, qualitative and quantitative differences in label distribution in lymphoid tissues. In mice given ^{14}C-chlorambucil, the thymus/blood ratio was 2.6 at 6 h after injection but there was less specific radioactivity after administration of ^{14}C-phenyl acetic mustard (thymus/blood ratio = 1.7).

Table 2. Tissue distribution of ^{14}C-ethyl groups in mice (means ± SD from ten animals)

Tissue	Time (h)	*p*.mole drug equivalent/mg			
		Chlorambucil	Ac-chlorambucil	Melphalan	Mitoclomine
Blood	0.5	101.0 ± 10.0	153.0 ± 8.0	11.5 ± 0.3	0.4 ± 0.08
	6	28.0 ± 2.0	45.5 ± 1.7	15.8 ± 0.4	1.7 ± 0.09
	12	21.8 ± 0.7	39.6 ± 1.7	21.5 ± 0.7	2.5 ± 0.16
	24	15.5 ± 1.0	27.1 ± 1.0	15.8 ± 0.2	3.2 ± 0.20
Liver	0.5	328.0 ± 25.0	257.0 ± 12.0	40.9 ± 2.0	6.9 ± 0.7
	6	81.8 ± 3.6	70.3 ± 1.7	41.6 ± 1.7	18.1 ± 1.0
	12	44.2 ± 1.7	37.3 ± 1.8	42.9 ± 2.0	19.1 ± 0.7
	24	30.7 ± 1.0	27.4 ± 2.3	21.1 ± 0.7	21.8 ± 0.6
Spleen	0.5	34.7 ± 2.6	44.5 ± 1.0	19.1 ± 2.6	2.1 ± 0.2
	6	26.4 ± 1.3	35.3 ± 1.0	23.1 ± 1.6	6.6 ± 0.3
	12	12.2 ± 0.3	22.1 ± 1.3	18.8 ± 0.7	6.9 ± 0.3
	24	8.9 ± 0.4	15.8 ± 0.6	16.5 ± 0.3	6.9 ± 0.4
Kidney	0.5	162.0 ± 20.0	152.0 ± 4.0	58.4 ± 2.6	5.0 ± 0.7
	6	118.0 ± 8.0	149.0 ± 2.0	84.8 ± 1.0	19.5 ± 1.6
	12	67.0 ± 1.3	86.1 ± 3.3	77.5 ± 3.0	18.8 ± 1.3
	24	46.2 ± 1.3	56.1 ± 2.3	48.5 ± 1.7	16.2 ± 0.5
Thymus	0.5	29.7 ± 2.3	38.6 ± 2.0	19.5 ± 1.9	0.8 ± 0.06
	6	71.9 ± 2.9	78.5 ± 3.3	19.8 ± 3.0	9.9 ± 0.7
	12	44.9 ± 1.6	40.6 ± 1.6	28.0 ± 1.0	12.5 ± 0.9
	24	29.4 ± 1.3	19.1 ± 1.0	19.0 ± 0.9	16.2 ± 0.3
Mesenteric lymph nodes	0.5	55.8 ± 4.3	59.4 ± 3.3	39.9 ± 4.9	4.8 ± 0.4
	6	32.7 ± 1.6	37.3 ± 0.7	32.3 ± 2.3	26.1 ± 2.9
	12	19.8 ± 1.0	25.4 ± 1.3	27.7 ± 2.6	28.7 ± 2.3
	24	12.5 ± 0.7	18.5 ± 1.0	18.1 ± 1.0	16.2 ± 1.3
Muscle	0.5	30.0 ± 2.3	35.0 ± 1.6	9.7 ± 0.7	0.17 ± 0.03
	6	9.7 ± 0.7	17.0 ± 0.7	14.3 ± 0.6	1.8 ± 0.1
	12	7.3 ± 0.4	14.0 ± 0.4	16.7 ± 0.5	3.3 ± 0.4
	24	7.3 ± 0.3	11.3 ± 0.6	19.3 ± 1.0	3.6 ± 0.3

As regards ^{14}C-melphalan, only mesenteric lymph nodes concentrated radioactivity during the hours immediately after drug injection. Following ^{14}C-mitoclomine administration, the thymus, lymph nodes, and to a lesser extent the spleen showed widespread label accumulation, and tissue/blood ratios were high (up to 5 for the thymus), indicating active uptake of some species carrying the ^{14}C-ethyl groups. No appreciable radioactivity was detected in the brain for any drug administered. All compounds were eliminated chiefly via the kidney (Table 3). Cumulative excretion of radioactivity over 48 h (urine and feces) in mice given ^{14}C-mitoclomine corresponded to less than 40% of the dose.

Table 3. Elimination of radioactivity in mice (percent dose) (mean ± SD from ten animals)

Cumulative excretion of	Time (h)	Urine	Feces
Chlorambucil	0–24	45.7 ± 2.0	6.5 ± 0.5
	0–48	54.2 ± 2.1	11.3 ± 0.8
Ac-chlorambucil	0–24	46.0 ± 1.9	2.0 ± 0.1
	0–48	53.0 ± 2.0	6.7 ± 0.3
Melphalan	0–24	53.6 ± 1.5	15.2 ± 1.1
	0–48	57.4 ± 1.4	18.7 ± 1.8
Mitoclomine	0–24	18.6 ± 1.0	4.8 ± 0.3
	0–48	27.3 ± 1.5	12.5 ± 0.7

Some of the most significant autoradiograms recorded are shown in Fig. 3. The low specific radioactivity of phenyl acetic mustard (0.3 μCi/mg) in relation to its toxicity did not allow us to obtain clear pictures compared with those for the other compounds.

Discussion

The small number of compounds included in this study does not permit mathematical analysis of their structure/activity relationships such as some authors

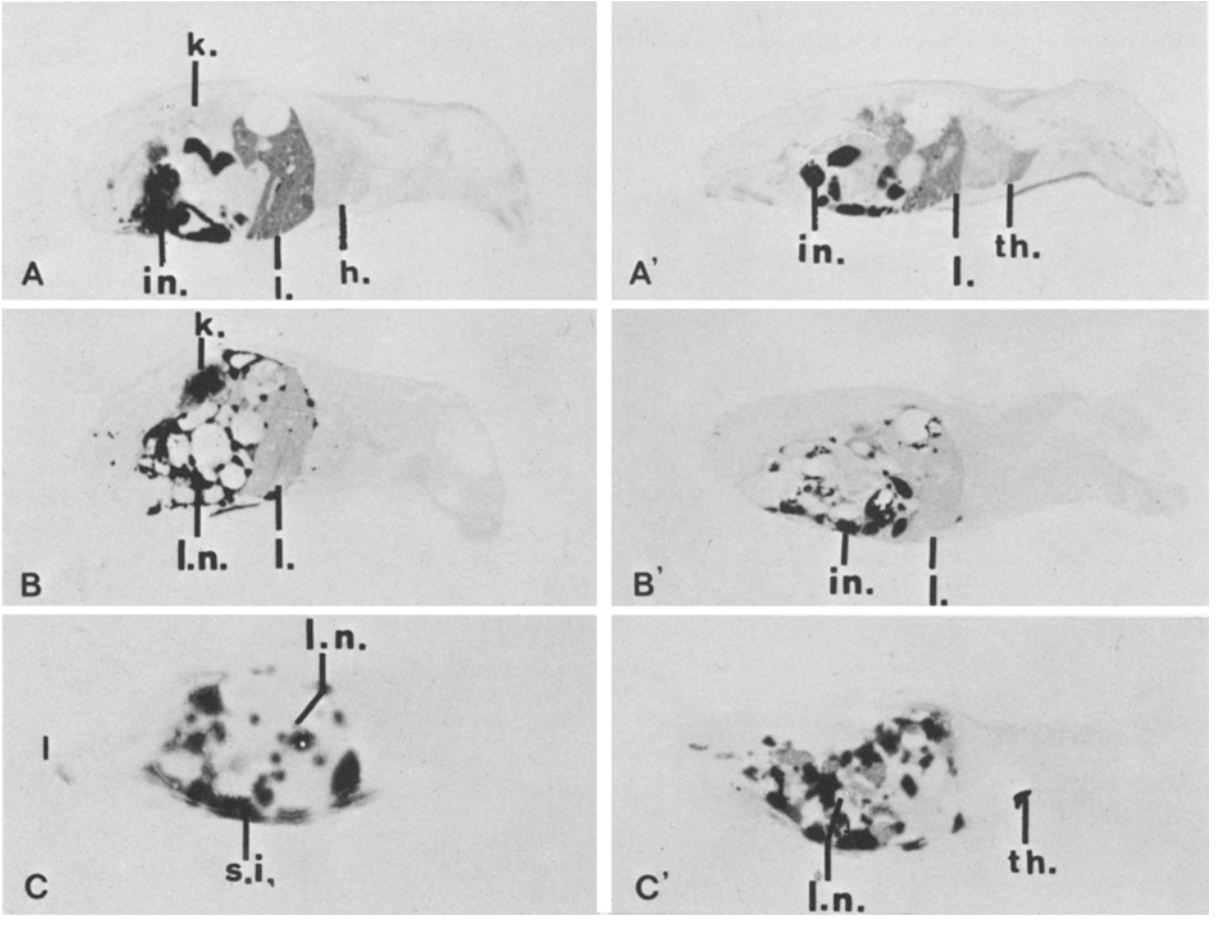


Fig. 3. Autoradiographs of mice at 0.5 h and 6 h after i.p. dosing with ^{14}C-ethyl-compounds. A,A', chlorambucil; B,B', melphalan; C,C' mitoclomine. l, liver; k, kidney; h, heart; in., intestine; l.n., lymph nodes; th. thymus; s.i., site of injection

have reported for other antineoplastic agents [2, 18, 19, 35, 40]. Nevertheless the present results call for several comments.

In this series of nitrogen mustards, the only parameter well correlated with toxicity as reflected by LD_{50} and antitumor activity was the partition coefficient. Phenylacetic mustard and melphalan, the most hydrosoluble compounds, proved the most toxic and were both also more effective in prolonging the survival of animals bearing Moloney's sarcoma. Toxicity and antitumor activity did not appear to be closely linked to alkylating activity. Mitoclomine, the most reactive compound towards *p*-nitrobenzylpyridine, proved the least toxic and also the least effective against Moloney's sarcoma. Alkylating activity of chlorambucil and phenylacetic mustard is significantly greater than that of melphalan. Nevertheless the latter compound, like phenylacetic mustard, showed the better efficiency in mice implanted with Moloney's sarcoma. These discrepancies can be attributed to very complex parameters involved in vivo, such as metabolism and effects peculiar to active metabolites. Panthananickal et al. [31] stated that the chemical and physicochemical properties of chlorambucil calculated or measured in vitro did not correspond with the drug efficiency in vivo. Its biological activity in fact derives from the resultant action of at least two alkylating entities, the parent compound and the more hydrophilic phenylacetic mustard. When the two drugs were tested against a solid tumor, the Walker carcinoma, the chemotherapeutic index of chlorambucil was found to be higher than that of its metabolite [22]. Moreover, authors have shown that the efficiency range of many related compounds was dependent on the tumor system used and on the site of implantation. Wodinsky et al. [41] obtained the same responsiveness (30%–33% ILS) to chlorambucil and melphalan in mice implanted IP with L1210 leukemia. Under these conditions, mitoclomine produces 64% ILS [M. Hayat, personal communication]. In contrast, melphalan produces a better response (75% ILS) than chlorambucil (17% ILS) when L1210 cells are implanted IV.

As reported for many antitumor agents, the mustards under investigation produced transitory inhibition of DNA synthesis in both normal and tumoral tissue but there were differences between the drugs in the timing of alterations, the degree of suppression, and the time required for recovery of normal DNA synthesis. Nevertheless, if the rapidly dividing cells of the bone marrow and intestinal mucosa are considered the most critical target sites, their return towards normal levels was undoubtedly faster than that of the tumor cells. It should also be stressed that the return to normal labeled precursor incorporation in tumor cells was faster after treatment with chlorambucil and mitoclomine. Moreover comparison of the percent ILS in mice bearing Moloney's sarcoma showed that these two drugs are also less effective in prolonging survival.

An attempt was made to investigate how far the chemical nature of the carrier affects the physiological disposition of the ethyl groups in animals. Although these studies were confined to measuring total radioactivity derived from ^{14}C-ethyl groups, results provided valuable indications of the distribution of the cytotoxic function. The very favorable partition coefficient of chlorambucil and phenylacetic mustard ($0 < \log P < 1$) should have led to absorption of these two compounds at a high rate. According to Penniston [32], such a coefficient facilitates passive drug transport through cell membranes. The early effect of these drugs observed with regard to DNA synthesis may be linked to their large-scale diffusion in the tissues very soon after administration. The apparently lower availability of melphalan, as reflected by the blood concentration of ^{14}C-ethyl groups, may be caused by a different absorption mechanism from that of chlorambucil and phenylacetic mustard. Vitisca et al. [38] recently demonstrated that in vitro and in vivo melphalan absorption dependend on an active or facilitated transport mechanism involving a high-affinity amino acid carrier of the L-leucine type. This process implies that transport sites can reach saturation and therefore that the rate of drug absorption might be limited. The strongly lipophilic character of mitoclomine seems to prevent it from being well distributed, as indicated by the retention of the label at the site of injection, the low blood radioactivity, and the elimination rate, which is distinctly lower than that of the three other compounds. The combined action of a high hydrolysis rate and poor cell penetration therefore endows mitoclomine with relatively weak toxicity.

No specific concentration of ^{14}C-melphalan in normal tissues was found except in the epuration and excretion organs (liver and kidney). This confirms the results reported by other authors who studied the biodisposition of melphalan labeled with ^{14}C on the alanine chain [6]. But the strong probability of a highly specialized transport mechanism if interesting to explore for its mechanism of action in myeloma, a disease characterized by an excessive protein synthesis in plasma cells and therefore by a greater need for amino acids or analogues [33].

Tissue distribution studies also suggest that the therapeutic effects of chlorambucil and mitoclomine in certain diseases affecting lymphocytes might be connected with the localization of cytotoxic groups in particular lymphoid areas. In the very first toxoco-

logical and pharmacological trials of chlorambicul in animals, Elson [18] found predominant lymphocytotoxic action in the thymus and hemapoietic bone marrow. Fox et al. [13] also found that in the rat, mitoclomine had a markedly depressive effect on the spleen, thymus, and lymph nodes, but a far more restricted one on the bone marrow.

More thorough investigations are needed to make sure that such selectivity on the part of chlorambucil, melphalan, and mitoclomine and/or their metabolites really result from the presence of a higher concentration of the active group in certain cell populations or subpopulations, and to determine the part played by certain carrying structures in a field which up till now has been little explored.

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