aniline (0.25 ml, 2.75 mmoles) in H_2O (2.5 ml); stirring was contd at room temp for ~5 hr. The ppt, washed with H_2O and dried *in vacuo* (P_2O_5), was identified (mp, tlc, ir) as carbanilide, yield 230 mg (49%).

Nitrosation of 73.—When $NaNO_2$ (255 mg, 3.70 mmoles) was added in portions to a cold (~8°), stirred soln of **73** (615 mg, 2.54 mmoles) in 98–100% HCO_2H (4 ml), a yellow ppt formed; the mixt was thinned with cold H_2O (10 ml) and stirred an addl 20 min. The ppt was collected on a fritted-glass filter, and, while still wet, half of it was immediately stirred in cold H_2O (10 ml) and treated with 40% aq $MeNH_2$ (0.5 ml). Immediate dissoln resulted followed by gradual pptn of a white solid, which, after 2–3 hr at room temp, was collected, dried *in vacuo* (P_2O_5), and identified as 1-methyl-3-phenylurea by ir comparison with a conventionally prepared sample: wt 135 mg (~71%); mp 140–141° dec. One recrystn from H_2O gave 45 mg (~24%), mp 147–149° (lit.[23] mp 151°). The other half of the nitrosated product decompd within 2 hr when stored over P_2O_5 in a desiccator at atm pressure.

1-Methoxy-3-(1,2,3,4-tetrahydro-2,4-dioxo-5-pyrimidinyl)urea (70).—A soln of $MeONH_2$, prepd by dissolving $MeONH_2 \cdot HCl$[55] (1.00 g, 12.0 mmoles) in 1 *N* NaOH (12 ml), was added to a stirred suspension of 1-methyl-1-nitroso-3-(1,2,3,4-tetrahydro-2,4-dioxo-5-pryimidinyl)urea[23] (2.55 g, 12.0 mmoles) in H_2O (100 ml). The mixt was warmed gradually, then refluxed for 1 hr, cooled to 50°, and filtered to remove insol matter. The filtrate was evapd to dryness *in vacuo*, and the residue was stirred with 1 *N* HCl (18 ml). The white product was washed with H_2O and dried *in vacuo* (P_2O_5) at 100° for 4 hr: yield 1.60 g (67%); λ_{max} in nm ($\epsilon \times 10^{-3}$) 267 (7.07) at pH 1, 267 (6.85) at pH 7, and 287 (6.25) at pH 13. (See Table VII.)

(2-Fluoroethyl)urea Nitrate (75).—Concd HNO_3 (4.57 ml) was added dropwise to a stirred paste consisting of (2-fluoroethyl)urea[4] (5.2 g, 49 mmoles) and H_2O (3.0 ml), and the resulting soln was chilled in an ice–salt bath. The crystals that formed were collected, dried *in vacuo* (P_2O_5), and recrystd from C_6H_6 (100 ml): yield 4.85 g (59%); mp 68–70°; ir (KBr) 1375 (s) and 825 (w) cm^{-1} (NO_3^-). *Anal.* ($C_3H_7FN_2O \cdot HNO_3$) C, H, N.

1-(2-Fluoroethyl)-3-nitrourea (76).—The nitrate **75** (3.50 g, 20.7 mmoles) was added in small portions to cold (−15 to −20°), stirred, concd H_2SO_4 (7.0 ml). After being stirred for 1 hr at −15°, the mixt was poured over ice–H_2O slush (35 ml), and stirring was contd at 0° for 1 hr. The cryst ppt was collected, washed with cold H_2O (3.5 ml), dried *in vacuo* (P_2O_5), and recrystd from C_6H_6 (~50 ml): yield 1.30 g (42%); mp 120°. A pilot run afforded the analytical sample: ir (KBr) 1600 and 1270 cm^{-1} (NO_2); pmr ($CDCl_3$) δ ~8 (**NH**) and ~11.5 (**NH**) ppm. (See Table IV).

1-(1-Adamantyl)-3-(2-fluoroethyl)urea (77) (from 76).—The nitrourea **76** (36 mg, 0.24 mmole) was added to a soln prepd by adding Et_3N (3 drops) and then Me_2CO (3 ml) to a soln of 1-adamantanamine·HCl[35] (45 mg, 0.24 mmole) in H_2O (3 ml). The mixt was heated at 70° for 1 hr, and the Me_2CO was evapd under reduced pressure. The pptd **77** was washed with H_2O and dried *in vacuo* (P_2O_5): yield 10 mg (17.5%); mp 212° (lit.[4] mp 212°); ir (KBr) 1610 (C=O), 1550 (CNH) cm^{-1}. The concd filtrate gave a negligible second crop.

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Selectivity of Action of Alkylating Agents and Drug Resistance. 4. Synthesis of Tritium-Labeled Chlorambucil and a Study of Its Cellular Uptake by Drug-Sensitive and Drug-Resistant Strains of the Yoshida Ascites Sarcoma *in Vitro*[1]

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The synthesis of ^3H-labeled chlorambucil is described and its uptake and utilization by drug-sensitive and drug-resistant strains of a Yoshida ascites sarcoma have been studied *in vitro*. Drug uptake is markedly influenced by the cell concentration and drug concentrations used. By selecting conditions similar to those achieved following *in vivo* drug treatment, the resistant cells have been shown, *in vitro*, to take up 50% less drug than the sensitive cells. This twofold difference in gross uptake of drug was also reflected in the absolute amounts of drug bound to protein. Chlorambucil appears to associate with an alcohol-soluble fraction of the Yoshida ascites cell, before extensive protein binding occurs. The fraction involved may be lipoprotein. It is unlikely that this represents a general reaction mechanism for all alkylating agents, since busulphan has been shown to combine directly with the intracellular protein of the cells.

A large number of neoplasms, both in man and experimental animals, appear to acquire resistance to treatment with alkylating agents following repeated exposure to these drugs: various mechanisms have been proposed to account for this. Several authors have detected an impaired transport of the drug by resistant cells,[2-4] though Wheeler and Alexander found that both drug-sensitive and drug-resistant plasmacytomas were equally effective in taking up cyclophosphamide,[5] while Novikova has demonstrated an enhanced uptake of phenylalanine mustard into several drug-resistant

(1) This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research, Royal Cancer Hospital) from the Medical Research Council and the British Empire Cancer Campaign for Research. One of us (B. T. H.) acknowledges the receipt of a Wellcome Foundation Postdoctoral Fellowship.

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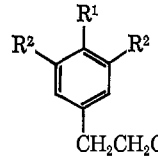
strains of sarcoma 45.[6] Other speculations concerning possible mechanisms of resistance include a selectively altered metabolism of the drug,[2] or a reduction in the extent of DNA alkylation as a result of "excision and repair" processes.[7,8]

Information concerning the gross uptake of alkylating agents and their subcellular localization in tumor cells remains sparse. Furthermore, as in our initial studies,[9] which showed no differences in the ability of the 2 strains of the Yoshida ascites sarcoma to accumulate a number of alkylating agents, the experimental conditions employed *in vitro* using very high drug concentrations often bear little resemblance to the *in vivo* situation. In an attempt to overcome some of these criticisms ^{3}H-labeled chlorambucil (Leukeran) has been synthesized and used to provide data on its cellular uptake and binding, utilizing physiological doses of drug.

Synthesis.—^{3}H-labeled chlorambucil, 4-(4-bis(2-chloroethyl)aminophenyl-*3,5-t*$_2$)butyric acid, was synthesized by a modification of the method of Wade and coworkers.[10]

Methyl 4-(4-aminophenyl)butyrate (I)[11] (Chart I) was

CHART I



I, $R^1 = NH_2$; $R^2 = H$; $R^3 = CH_3$
II, $R^1 = NH_2$; $R^2 = I$; $R^3 = CH_3$
III, $R^1 = N(CH_2CH_2Cl)_2$; $R_2 = I$; $R^3 = CH_3$
IV, $R^1 = N(CH_2CH_2Cl)_2$; $R^2 = I$; $R^3 = H$
V, $R^1 = N(CH_2CH_2Cl)_2$; $R^2 = H$; $R^3 = H$

converted into the 3,5-diiodo derivative II by brief (5 min) reaction with ICl in concd HCl, which was then converted into the aromatic mustard III by the method of Everett, *et al.*[11] Acidic hydrolysis, the subsequent step in the synthesis by these workers, could not be used to remove the Me from IV, owing to its low solubility in HCl. However, the OH^- catalyzed hydrolysis of the ester grouping should proceed by an SN2 mechanism,[12] in contrast to the SN1 process which is the rate-determining step in the hydrolysis of *N*-(2-chloroethyl) groups.[13] This mechanistic difference was exploited for the selective hydrolysis of the Me ester group, using $(n\text{-Bu})_4N^+OH^-$ in a nonpolar medium of MeOH–PhH which disfavored the SN1 process.

The reductive tritiation of the hydrolysis product IV in the presence of Et_3N was followed by the acidification of the reaction mixture to remove labile ^{3}H and to liberate the free acid V from its triethylammonium salt. The theoretical volume of ^{3}H was consumed, but the radioactive content of the organic phase after acidic treatment was only 11% of the theoretical value. This deficiency could have resulted from the incorporation of H atoms derived from the unprotected CO_2H of V into the ^{3}H, and subsequent H–halogen exchange. Wade and Murphy[10] considered in their synthesis of tritiated melphalan that vigorous acidic treatment (6 *N* HCl, 4- to 5-hr reflux) was a major cause of loss of ^{3}H from positions ortho to an amine substituent. The milder acidic treatment used here to extract the free acid V may be responsible for this observed loss of activity and then the residual activity would be due not to ^{3}H substituted in the positions shown in structure V, but to the incorporation of ^{3}H into the positions meta to the substituted amino group by a mechanism other than direct ^{3}H–halogen exchange.

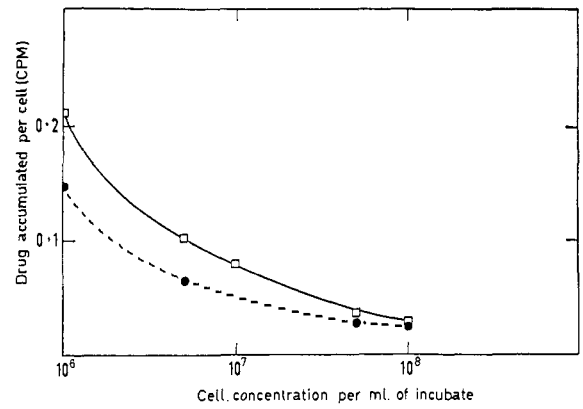


Figure 1.—The effect of various cell concns on the uptake of chlorambucil by Yoshida ascites cells *in vitro:* chlorambucil concn, 0.02 *M;* (□—□) sensitive cells; (●----●) resistant cells.

The specific activity (183 mCi/mole) of the cryst material obtained after dilution of the tritiated product with nonradioactive chlorambucil was sufficient for these initial studies. Ball and Wade[14] have synthesized aniline mustard of very high specific activity, using a meta-substituted iodo derivative to ensure the stability of the incorporated ^{3}H. The synthesis of an analogous chlorambucil derivative is now being investigated.

In Vitro **Metabolic Studies.**—The methods used are described in detail in the Experimental Section. In a previous report we used a colorimetric method to follow the pattern of uptake of chlorambucil by Yoshida ascites cells.[9] Because of the low sensitivity of this analytical technique it was necessary to expose high concentrations of cells to high drug concentrations. With this preparation of chlorambucil-*t* the kinetics of drug uptake have been followed in greater detail and under more physiological conditions.

Differences in the extent of drug uptake and subsequent metabolism have been observed, depending on the cell concentrations and drug concentrations employed for *in vitro* incubations. The results of a series of experimental findings are shown in Figure 1 and listed in Table I. Drug uptake is markedly influenced by the number of cells present. Over a range of cell concentrations (10^6–10^8 cells/ml) and at a fixed drug concentration (0.02 *M*), the amount of drug retained by cells of each strain fell progressively, and approached a common value as the cell number was raised (Figure 1). At 10^6 cells/ml the sensitive cells contained approximately 50% more drug than the resistant cells, while

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TABLE I

AMOUNT OF CHLORAMBUCIL ACCUMULATED BY DRUG-SENSITIVE AND -RESISTANT STRAINS OF THE YOSHIDA ASCITES SARCOMA, FOLLOWING *in Vitro* INCUBATION AT 37° WITH VARYING DRUG CONCENTRATIONS

Cell concn, per ml	Drug concn, m*M*	Gross drug accumulated after 30-min incubation, dpm per cell		% drug accumulated by resistant cells as compared with sensitive cells
		Resistant	Sensitive	
10^7	0.03	0.023	0.044	52
10^7	0.08	0.043	0.082	52
10^7	0.17	0.065	0.133	51
10^7	0.33	0.109	0.216	60
10^7	1.00	0.387	0.460	85
10^7	1.66	0.812	0.920	89

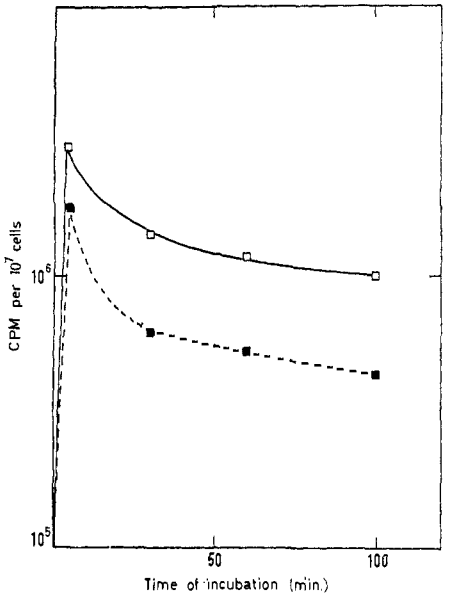

Figure 2.—The pattern of uptake of chlorambucil into Yoshida ascites cells: (□—□) sensitive cells; (■----■) resistant cells. The cells were incubated with 0.33 *M* chlorambucil at a concn of 10^7 cells per ml at 37° for 100 min. Drug uptake was followed as described under Methods in the text.

at 10^8 cells/ml, both strains contained comparable amounts. This behavior might be explained by the presence of a greater number of drug-binding sites on the sensitive cells, and that access to these sites is limited by increasingly close physical contact of the cells as their concentration is increased.

The amount of chlorambucil accumulated by the cells was linearly dependent on its concentration below 0.33 m*M* (Table I), though at higher drug concentrations both cells incorporated equiv amounts of drug. At 3.3 m*M* the cells were visibly damaged and it was impossible to obtain quant data. It seemed likely, therefore, that results obtained using concentrations in excess of 0.33 m*M* were associated with membrane damage.

These results emphasize that in assessing the relative abilities of drug-sensitive and -resistant tumor cell strains to incorporate chlorambucil, the cell concentrations and drug concentrations used in *in vitro* incubation experiments are critical. Therefore conditions were selected similar to those achieved following *in vivo* drug treatment, namely 10^7 cells/ml and drug concentrations of 0.002 m*M* and 0.2 m*M*. These doses represented the minimum levels necessary to produce a lethal effect on the sensitive and resistant strains of cells, respectively, *i.e.*, the cells were nonviable on subsequent transplantation into healthy animals. Figure 2 shows that using a drug concentration of 0.2 m*M*, chlorambucil is taken up rapidly by both sensitive and resistant cells, and after 5 min it is possible to detect a progressive loss of label from cells of both strains, which leads at 100 min to the presence of approximately twice as much drug in the sensitive cells as in the resistant cells. Similar results were obtained using a drug concentration of 0.002 m*M*, except that the drug taken up by the 2 cell strains was correspondingly diminished.

Details of the binding of chlorambucil (0.2 m*M*) to ascites cells following its uptake were investigated using 2 protein-precipitating agents, namely EtOH and 10% TCA (Cl_3CCO_2H). The results of these studies are shown in Figure 3. When EtOH was used as the protein precipitant only 6% of the drug entering the cells during the first 5 min of incubation, was found in the EtOH-insol pellet. The amount of protein-bound drug increased with time, and after 100 min, 70% of the total drug within the cells was protein bound, in both cell strains. Consistent with these findings the amount of drug retained in the EtOH-soluble fraction decreased with time. However, different results were obtained when TCA was used as a protein precipitant. After an initial 5-min incubation, approximately 60% of the accumulated drug was found in the TCA-insol fraction. Furthermore, the drug retained in both the TCA-sol and TCA-insol extract decreased with time. At 100 min most of the drug was protein bound in both cell strains.

To summarize the results of Figure 3: the total amount of drug accumulated by the whole cells decreased with time. The amount bound to the EtOH-insol ppt increased with time, while the amount bound to the TCA-insoluble ppt decreased over the 100-min incubation period. Irrespective of the extractant used, however, the same quantity of drug remained bound to protein at 100 min. The distribution of the drug between sol and insol fractions was similar for both sensitive and resistant tumor cells. However, only 15% of the drug initially taken up by resistant cells remains bound to protein after 100 min compared with 26% in the sensitive cells. Therefore, the resistant cells not only accumulated less drug than their sensitive counterparts, but also the extent of drug-protein binding was lower.

The following experiment was performed in an attempt to account for the differences shown in Figure 3. After incubation with chlorambucil, cells were extracted (a) with TCA alone, (b) with EtOH alone, (c) with TCA followed by EtOH. It was found that approximately 80% of the counts associated with the TCA-insol fractions at 5 min (*ca.* 10^6 dpm per 10^7) could be removed by a subsequent extraction with EtOH. The resultant pellet then carried a similar number of counts as one obtained from a single EtOH extraction (*ca.* 2×10^5 dpm per 10^7 cells). These observations may be associated with the presence of lipoprotein in the EtOH-sol extract, in contrast to the situation with TCA, when lipoprotein was pptd with the total cellular protein.

To investigate further this difference in action of the 2 protein-precipitating agents, and to show whether this was specific for chlorambucil, similar incubation experiments were carried out using ^{3}H-labeled busulphan, 1,4-bis(methanesulfonyloxy)butane-*2,3-t*$_2$ (specific activity 8.0 Ci/mmole),[15] at 10^{-6} *M* in the incubate. The results of these studies are shown in Figure 4. It is apparent that both tumor cell lines accumulated comparable quantities of the drug, and that EtOH and TCA behaved similarly as protein-precipitating agents in this system. Furthermore, there was little alteration in the quantities of drug held in the insol phase during the 100-min incubation period, while the amount of drug bound to protein increased approximately 2.5-fold.

This differential distribution of chlorambucil, following its accumulation by the cells, into the EtOH-sol and -insol extracts may possibly be attributed to an immediate association of the drug with lipoprotein components, followed by a slow release from this union as the drug alkylates protein. It is likely that there are individual differences between the alkylating agents in the mechanism of drug–protein interaction *in vitro*, as shown by these present results, which may also be of importance *in vivo*. Linford,[16] working with red blood cells, has proposed that the chlorethyl groups of chlorambucil are fat soluble and are involved in the process of absorption of the agent at the cell membrane. Our results tend to support these conclusions. However, the presence of the aliphatic side chain in chlorambucil should not be overlooked when assessing the drug's lipophylic properties.

Differing patterns of uptake and metabolism of various alkylating agents have also been revealed in other studies. Gati and Koros[17] have ascribed degranol resistance in a strain of NK/lymphoma cells to an enhanced ability of the cells to bind the drug. Furthermore, Novikova[18] isolated 10 times more sarcolysin bound to the DNA of a resistant transplantable sarcoma 45 than could be detected in the DNA of a sensitive tumor. These findings, together with those presented above, imply that though there might be many reactions common to all alkylating agents (*e.g.*, alkylation of the DNA strands), there must in addition be discrete biochemical reactions which contribute to the individual pharmacologic effects of these agents, and that transport of the drug and its interactions with macromolecular components of the cell may have some relevance in determining the mechanism of resistance.

Experimental Section[19]

Methyl 4-(4-Amino-3,5-diiodophenyl)butyrate.—Solns of methyl 4-(4-amino)butyrate[11] (9 g) in concd HCl (50 ml) and of ICl (26 g) in the same solvent (20 ml) were mixed at room temp. After 5 min, water (500 ml) was added, then 10 min later the mixt was extd with EtOAc (500 ml). The dark org phase was washed with satd aq $NaHSO_3$ (250 ml) to remove I_2, and the yellow soln dried ($MgSO_4$) and concd under reduced pressure. A soln of the residue of PhH (100 ml) was applied to a silicic acid column (20 cm × 8 cm^2) and eluted with PhH (1 l.). On crystn of the concd PhH fractions from petr ether (bp 80–100°, 200 ml) the product formed colorless needles (8.2 g, 40%), mp 90–91°. *Anal.* ($C_{11}H_{13}I_2NO_2$) C, H, I, N.

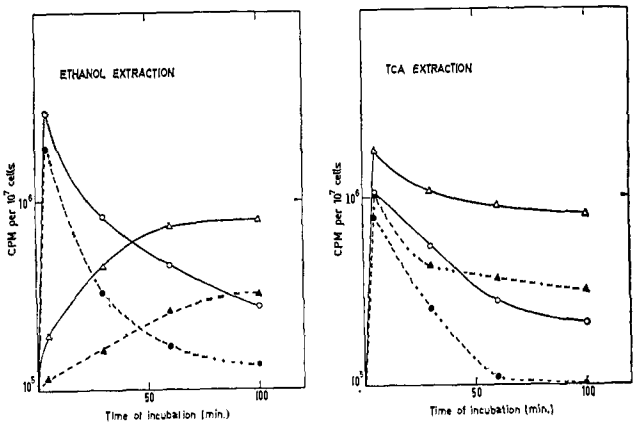

Figure 3.—The distribution of chlorambucil, following its uptake, between drug-sensitive and -resistant Yoshida ascites cells, using two protein-precipitating agents, EtOH and TCA: (Δ—Δ) sensitive insol extract; (▲----▲) resistant insol extract; (○—○) sensitive sol extract; (●----●) resistant sol extract.

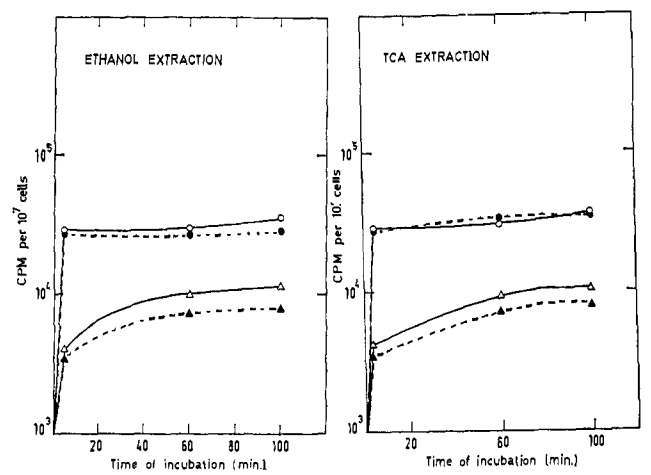

Figure 4.—The distribution of busulphan, following its uptake, between drug-sensitive and -resistant Yoshida ascites cells, using 2 protein-precipitating agents, EtOH and TCA: (Δ—Δ) sensitive insol extract; ▲----▲) resistant insol extract; (○——○) sensitive sol extract; (●----●) resistant sol extract.

Methyl 4-(4-Bis(2-chloroethyl)amino-3,5-diiodophenyl)butyrate.—Methyl 4-(4-amino-3,5-diiodophenyl)butyrate (5 g) dissolved after stirring for 1 hr at room temp in a soln of ethylene oxide (10 g) in glacial AcOH–H_2O (9:1 v/v) (50 ml). After 16 hr the soln was concd under reduced pressure and the residue was partitioned between PhH (150 ml) and satd aq $NaHCO_3$ (50 ml). The org phase was dried ($MgSO_4$) and 50 ml of PhH removed by distn. $POCl_3$ (10 ml) was added and the soln refluxed for 1 hr and finally concd under reduced pressure. The residue was partitioned between Et_2O (50 ml) and satd aq $NaHCO_3$ (50 ml). The dried ($MgSO_4$) org phase was concd, and a soln of the residue in CH_2Cl_2 was applied to a column of silicic acid (15 cm × 8 cm^2) which was eluted with the same solvent (20-ml fractions). On crystn, after concn, of fractions 4–10 from petr ether (bp 80–100°, 50 ml), the product formed colorless square plates (3.5 g, 55%), mp 56–57°. *Anal.* ($C_{15}H_{19}Cl_2I_2NO_2$) C, H, Cl, I, N.

4-(4-Bis(2-chloroethyl)amino-3,5-diiodophenyl)butyric Acid.—A soln of methyl 4-(4-bis(2-chloroethyl)amino-3,5-diiodophenyl)-butyrate (5 g) in *n*-$Bu_4N^+OH^-$ in MeOH–PhH (0.1 *M*, 100 ml) was refluxed for 2 hr. The cooled soln was shaken with HCl (1 *N*, 100 ml). The org phase was dried ($MgSO_4$). On crystn, after concn, from PhH–petr ether (bp 80–100°) (1:2) (40 ml), the product gave colorless needles (4.25 g, 87%), mp 120–121°. *Anal.* ($C_{14}H_{17}Cl_2I_2NO_2$) C, H, Cl, I, N.

(15) Tritium-labeled busulphan (Myleran) was purchased from Schwarz BioResearch Inc., New York, N. Y.

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(18) M. A. Novikova, VIII International Cancer Congress, Moscow, 1962, p 336.

(19) Melting points, which were corrected, were determined with a Kofler hot-stage apparatus.

4-(4-Bis(2-chloroethyl)aminophenyl)butyric Acid (Chlorambucil).—A soln of 4-(4-bis(2-chloroethyl)amino-3,5-diiodophenyl)butyric acid (0.556 g) in dry dioxane (5 ml) containing Et_3N (0.3 ml) and 5% Pd/C (0.15 g) was stirred, in the dark, under H_2 overnight at room temp. The theoretical vol (45 ml) was consumed. The catalyst was filtered off (Hyflo) and washed with EtOAc (10 ml) and the combined filtrates were treated successively with HCl (1 *N*, 10 ml) and H_2O (10 ml). The org phase was dried ($MgSO_4$). On crystn, after concn, from PhMe–petr ether (bp 30–40°), 1:1 (3 ml) at −20°, the product (0.19 g) formed colorless crystals, mp 64–66°, identical with the published value.[11]

Reductive Titration of 4-(4-Bis(2-chloroethyl)amino-3,5-diiodophenyl)butyric Acid.—Tritiation was carried out by the above reductive dehalogenation procedure, using $^3H_2$, and the same quantities of reagents, except that 0.2 g of the diiodo derivative was employed. The theoretical vol (17 ml, 40 Ci) of $^3H_2$ was consumed in 24 hr. The filtered soln was dild with EtOAc (10 ml) and extd with HCl (1 *N*, 10 ml) to remove labile ^3H, and to liberate the free acid from its $NHEt_3$ salt, then with H_2O (10 ml). The dried ($MgSO_4$) soln was dild to 100 ml with EtOAc. The total activity was 2.19 Ci (11% of the theoretical value). The soln was concd at 30° under reduced pressure and, after the addn of 0.5 g of nonradioactive chlorambucil, the residue was crystd at −20° from PhMe–petr ether (bp 30–40°), 1:1 (10 ml). The yield was 0.369 g, with a specific activity of 183 mCi/mmole. The product was stored in the dark in soln in PhH (50 ml) at room temp.

Radiochemical Purity and Stability of Tritiated Chlorambucil.—The soln was assayed for radiochemical purity after storage for 8 months. A sample of the PhH soln was chromatographed alongside nonradioactive chlorambucil on plates coated with silica gel, using MeOH–$CHCl_3$ (3:25) as the developing solvent. Portions were scraped off at 1-cm intervals into HCl (0.1 *N*, 0.3 ml) and, after standing overnight, dild with phosphor (10 ml) and assayed by scintillation counting; 88% of the radioactivity occurred in the region corresponding to the authentic material; 8% of the total activity on the plate was present in the region of the origin.

The origin counts could be removed by application of the PhH soln of the product (1 ml) to a column of silicic acid (5 cm × 0.75 cm^2) and elution with $CHCl_3$ (10 ml). The eluate was concd and the residue was dissolved in PhH. One such soln, containing 50 mCi/l., prepd from the soln of original product after 5-months storage, was assayed for radiochemical purity as above after storage in the dark for a further 3 months at room temp; 96% of the radioactivity occurred in the region corresponding to the authentic material. Less than 1% of the total activity on the plate was present in the region of the origin, indicating that the original origin counts were due to unremoved labile ^3H in the cryst batch and not to subsequent radiolysis of the PhH soln on storage.

Animals.—Full details of the animal experimentation and tumor transplantation techniques have been given previously.[20,21] The animals were killed by cervical dislocation in the 5th–6th day after tumor transplantation and the peritoneal contents were aspirated with 5 ml of a 3% solution of PBS (phosphate buffered saline). The cells were removed from suspension by centrifugation at 500*g*, 4°, washed in PBS, and resuspended to a known vol. The cell concn was detd using an electronic particle counter (Model A, Coulter Electronics, Kenmore, Chicago, Ill.) with threshold and aperture current settings 15 and 2, respectively.

Cell Suspensions.—The cell concns were adjusted to 10^7 cell/ml with PBS, and 12 ml of this suspension was transferred to 25-ml stoppered flasks and allowed to equilibrate in a metabolic shaker (Gallenkamp) at 37° for 10 min; 0.1 ml of chlorambucil-*t* in EtOH (6.0 mg/ml and 2 mCi/ml) or 0.1 ml of busulphan-*t* (0.03 mg/ml and 1 mCi/ml).

Drug uptake was followed by withdrawing 2-ml aliquots of cell suspensions at measured time intervals after drug addn. Similar samples were taken from control cell suspensions which had been treated with solvent only. Cells were removed from the sample suspensions by centrifugation at 500*g* (4°) and washed twice with 2-ml vols of PBS. The resultant cell pellets were then either (a) dissolved in 12.5% aq $Et_4N^+OH^-$ (for measurements of gross drug uptake by the cells), or (b) extd by shaking for 1 min successively with two 2-ml vols of either EtOH or 10% TCA. After each extn the samples were left to stand at 0° for 30 min before removing the supernatant fluid. The combined TCA or EtOH supernatant fractions were then stored and the final ppts were digested, either in 1 ml of 12.5% aq Et_4NOH or in 1 ml of 1 *N* NaOH.

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