Biochemical Pharmacology, Vol. 27, pp. 151–166. Pergamon Press, 1978. Printed in Great Britain.

MALEIMIDE—BIOCHEMICAL, PHARMACOLOGIC AND TOXICOLOGIC STUDIES

INTERACTION WITH L-ASPARAGINE METABOLISM*

DAVID A. COONEY,† HARRY A. MILMAN, RITCHARD G. CABLE
ROBERT L. DION and VINCENT H. BONO, JR.
Laboratory of Toxicology (D.A.C., H.A.M. and R.G.C.),
Molecular Biology and Methods Development Section (R.L.D. and V.H.B.), Drug Evaluation Branch,
National Cancer Institute, National Institutes of Health Bethesda, MD 20014, U.S.A.

and

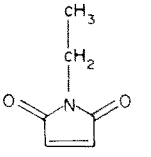
KARL KARRER and HANS P. FRIEDL
Institute for Cancer Research of the University of Vienna, Wien IX, Borschkegasse 8A, Austria

(*Received* 18 *August* 1976; *accepted* 27 *April* 1977)

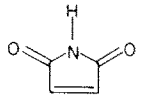
Abstract– The effect of maleimide on the metabolism of L-asparagine has been examined *in vitro* and *in vivo*. *In vitro*, maleimide is a potent and irreversible inhibitor of the L-asparagine synthetase [L-glutamine hydrolyzing, (EC 6.3.5.4)] from murine leukemia 5178Y/AR and from murine pancreas; this inhibition is accomplished via sulfhydryl blockade and can be prevented by suitable thiols. *In vivo*, maleimide was highly irritating locally, producing peritonitis and phlebitis after intraperitoneal and intravenous injection, respectively. The LD_{50} in the mouse was 9 mg/kg by the intravenous and intraperitoneal routes; renal, hepatic, neurologic and hematologic toxicities were the principal effects of the drug in this species. L-Asparagine did not alter the lethal effects of maleimide. Maleimide at a dose of 6 mg/kg intraperitoneally did not begin to inhibit pancreatic or tumoral L-asparagine synthetase until 24 hr after its administration; maximum inhibition was delayed until 48–72 hr after dosing. Although maleimide was found to be a potent inhibitor of hepatic L-asparaginase (EC 3.5.1.1) and L-asparagine transaminase (EC 2.6.1.14) *in vitro*, at no time did the agent inhibit these enzymes *in vivo*. Additionally, maleimide failed to inhibit protein and nucleic acid synthesis in pancreas and liver, although it did exert a transient repressive effect on these processes in subcutaneous L5178Y/AR tumor. The drug was a potent cytotoxin to L1210 cells in culture, causing partial arrest in the G_2 phase of the cell cycle as well as general slowing of progression through the cell cycle. No therapeutic action was produced vs this tumor growing in the peritoneum of mice nor was the drug effective vs subcutaneous L5178Y or L5178Y/AR. However, when maleimide was used to wash the surgical wound created by extirpation of Lewis Lung Carcinoma growing in the muscles of the hind limb, the drug did inhibit local recurrence of tumor, most notably when used in conjunction with parenteral cyclophosphamide. It is concluded that maleimide is an inhibitor, *in vitro*, of the enzymes of L-asparagine metabolism because of its ability to form covalent bonds with critical sulfhydryl compounds.

N-ethyl maleimide (NEM) (Fig. 1a) is a widely used biochemical reagent known for its ability to react with sulfhydryl functions. Maleimide (Fig. 1b), bearing the same reactive double bonds, is also a powerful sulfhydryl reagent. Moreover, maleimide shows a resemblance to the postulated cyclic anhydride of L-asparagine [1, 2]. The interaction of maleimide with the enzymes of L-asparagine metabolism, therefore, may be hypothesized to occur in one of two ways: via sulfhydryl-alkylation (in the event that these enzymes possess crucial thiol functions), and/or as a substrate analogue (in the event that cyclization of L-asparagine supervenes in the course of catalysis).

To test these hypotheses, the interaction of maleimide with L-asparagine synthetase, L-asparagine α-keto acid transaminase and several L-asparagine



(a) *N*-Ethyl maleimide



(b) Maleimide

Fig. 1. Structures of *N*-ethyl maleimide and maleimide.

* This work is taken in part from a dissertation by Harry A. Milman to be presented to the Department of Pharmacology, The Graduate School of Arts and Sciences, The George Washington University, in partial fulfillment of the requirements for the Ph.D.

† Send reprint requests to: Dr. David A. Cooney, Laboratory of Toxicology, Building 37, Room 5B22, National Cancer Institute, National Institutes of Health, Bethesda, MD 20014.

amidohydrolases has been examined *in vitro*. Furthermore, in order to permit the safe use of maleimide *in vivo*, certain toxicologic features of the agent have been delineated. Finally, the oncolytic and enzymologic properties of maleimide have been examined in the intact tumor-bearing mouse.

MATERIALS AND METHODS

Enzymes

L-Glutamate-oxaloacetate transaminase (GOT) (EC 2.5.1.1, sp. act. 180 I.U./mg of protein), malate dehydrogenase (MDH) (EC 1.1.1.37, sp. act. 1100 I.U./mg of protein), L-glutamate-pyruvate transaminase (GPT) (EC 2.5.1.2, sp. act. 80 I.U./mg of protein), L-glutamate dehydrogenase (GDH) (EC 1.4.1.2, sp. act. 45 I.U./mg of protein), lactic acid dehydrogenase (LDH) (EC 1.1.2.3, sp. act. 360 I.U./mg of protein), and catalase (EC 1.11.1.6, sp. act. 39,000 I.U./mg of protein) were purchased from Boehringer-Mannheim, New York, NY, U.S.A. L-Glutamate decarboxylase (EC 4.1.1.15, sp. act. 10 I.U./ml) and L-aspartate-β-decarboxylase (EC 4.1.1.12, sp. act. 77 I.U./mg of protein) were purified as previously described [3].

Chemicals

Maleimide, *N*-ethyl maleimide (NEM), and maleamic acid, were the products of Aldrich Chemicals, Cedar Knolls, NJ, U.S.A. L-Asparagine and reduced nicotinamide adenine dinucleotide (NADH) were acquired from Sigma, St. Louis, MO, U.S.A. α-Ketoglutaric acid, α-ketovaleric acid and dithiothreitol were procured from Calbiochem, Gaithersburg, MD, U.S.A. Showdomycin [1-*H*-pyrrole-2,5-dione,3-β-D-ribofuranosyl-, (NSC-93047)] was obtained from the Division of Cancer Treatment of the National Cancer Institute, Bethesda, MD, U.S.A. Electrophoretic analysis showed it to be free of maleimide. Other substituted maleamic acids and maleimides were synthesized by Dr. J. Althaus of this laboratory according to the general procedures of Welman *et al.* [4]. Substituted furanone derivatives were kindly donated by Dr. F. Farina from the Instituto de Quimica Organica General, Madrid, Spain.

Radiochemicals

L-[4-^{14}C]aspartic acid (sp. act. 22.2 μCi/μmole) was the product of Biochemical and Nuclear Corp., Burbank, CA, U.S.A. L-[U-^{14}C]asparagine (sp. act. 151 μCi/μmole) was the product of Amersham Corp., Arlington Heights, IL, U.S.A. Purity of these chemicals was assessed as previously described [5]. L-(U-^{14}C]valine (sp. act. 225 μCi/μmole) was purchased from New England Nuclear, Boston, MA, U.S.A.

Vessels

Eppendorf 1500-μl plastic centrifuge vessels procured from the Brinkman Instrument Co., Silver Spring, MD, U.S.A., were used throughout.

Tumor

Leukemia 5178Y was maintained by serial passage in BDF_1 mice. Resistance to L-asparaginase was achieved by treatment of the mice with subcurative doses of L-asparaginase as previously described [5].

Tissue preparation

For enzyme assays on tissue homogenates, BDF_1 mice were killed by cervical dislocation, relevant organs were immediately collected and homogenized in 3 vol. (w/v) of 0.05 M Tris–HCl buffer, pH 8.4, or in 0.1 M Tris–HCl buffer, pH 7.6, containing 1 mM dithiothreitol (DTT) and 0.5 mM disodium-EDTA (for pancreatic homogenates only). The crude homogenates were transferred to Eppendorf 1500-μl conical vessels, centrifuged at 12,000 g for 6 min, after which the supernatants were transferred to fresh Eppendorf 1500-μl conical vessels and stored at −87° until use.

Preparation of microsomal fractions

Hepatic microsomal fractions from BDF_1 mice were prepared by the method of Gram *et al.* [6]. Assays of microsomal enzyme activity were performed immediately.

Enzyme assays

L-Asparagine synthetase. L-Asparagine synthetase (L-glutamine hydrolyzing, EC 6.3.5.4) was measured on the 12,000 g supernatant of organ homogenates described above (crude enzyme source) or on 3-fold purified enzyme from mouse pancreas or L5178Y/AR. Partial purification was achieved by supplementing the extracts with ATP, $MgCl_2$ and NH_4Cl to final concentrations of 0.01 M, and heating at 50° for exactly 10 min. To the 20,000 g (10 min) supernatant from this step, solid $(NH_4)_2SO_4$ was added to 30% of saturation and the precipitate discarded. L-Asparagine synthetase then was salted out from the supernatant by the addition of solid $(NH_4)_2SO_4$ to 50% of saturation. The resulting pellet was taken up in a small amount of the homogenizing buffer, dialyzed for 3 hr against three changes of 0.05 M Tris HCl buffer, pH 8.4, and analyzed at once. The partially purified enzyme was used only for the measurement of inhibition constants.

A radiometric technique was used to measure enzyme activity. In a final volume of 45 μl were admixed 0.003 μmole L-[4-^{14}C]aspartic acid (sp. act. 22.2 μCi/μmole), 1 μmole ATP, 1 μmole $MgCl_2$, 0.9 μmole L-glutamine or 2.5 μmole NH_4Cl and 2.5 μmole Tris–HCl buffer, pH 8.4, along with 10 μl of the enzyme source to be assayed. In some experiments (see legends to appropriate tables and figures), a 15-μl incubation mixture was used which contained 5.6 nmoles (0.125 μCi) L-[4-^{14}C]aspartic acid, 0.2 μmole L-glutamine or 0.5 μmole NH_4Cl, 0.1 μmole ATP, 0.25 μmole $MgCl_2$ and 2.5 μmoles Tris–HCl buffer, pH 7.6, along with 5 μl of the enzyme to be assayed.

After a 30-min incubation at 37°, during which time the synthesis of L-[4-^{14}C]asparagine was found to be linear, the vessels were heated at 95° for 5 min to terminate the reaction, then centrifuged at 12,000 g for 3 min to precipitate denatured protein. Subsequent removal of unreacted L-[4-^{14}C]aspartic acid and the recovery of synthesized L-[4-^{14}C]asparagine were conducted by the methods previously described [5]. Suitable blanks and standards were included in each assay to verify that the product synthesized was L-asparagine.

L-Asparagine transaminase. L-Asparagine transaminase (EC 2.6.1.14) was extracted from mouse liver

as described above. The homogenizing medium was 0.05 M Tris–HCl buffer, pH 7.6, containing 0.5 mM disodium-EDTA and 1 mM dithiothreitol. The supernatant was dialyzed overnight at 4° against 4 l. of homogenization buffer lacking DTT, but supplemented with 20% (v/v glycerol. Measurement of enzyme activity was conducted radiometrically as follows. In a final volume of 20 μl were admixed 5 μl (0.25 μCi) L-[U-^{14}C]asparagine, 5 μl of 0.05 M Tris–HCl buffer, pH 8.4, 5 μl of 0.03 M α-ketovaleric acid in 0.05 M Tris-HCl buffer, pH 8.4, or of 0.05 M Tris–HCl buffer, pH 8.4, and 5 μl of the crude transaminase. After 30 min at 37°, any [U-^{14}C]α-ketosuccinamic acid was α-decarboxylated by the addition to the reaction mixtures of 50 μl of 1% (v/v) H_2O_2 in 1 M HCl. The [^{14}C]O_2 so generated was trapped in a droplet of 40% (w/v) KOH deposited on the underside of the lid of the closed vessel and counted as previously described [7].

L-Asparaginase. L-Asparaginase (EC 3.5.1.1) was extracted from mouse liver and the supernatant dialyzed as described for L-asparagine transaminase. A radiometric technique was used to quantitate the hydrolysis of L-asparagine, as follows. Five μl of a freshly prepared maleimide solution of the requisite molarity in 0.1 M Tris–HCl buffer, pH 8.4, or 5 μl of 0.1 M Tris–HCl buffer, pH 8.4, and 5 μl L-asparaginase solution were driven onto a 5-μl droplet (0.25 μCi) of L-[U-^{14}C]asparagine contained in the bottom of an Eppendorf 1500-μl centrifuge vessel by a 5-sec acceleration to 12,000 *g*. After a 30-min incubation at 25°, 10 μl of 1 M HCl was added to each vessel and the vessels were heated at 95° for 5 min. Ten μl of 1 M NaOH then was added to each vessel followed by 20 μl of a decarboxylation mixture consisting of 6.8 mM α-ketoglutaric acid, 8.5 mM $ZnSO_4$ in 0.66 M sodium acetate buffer, pH 5.0, containing 20 I.U. GOT/ml. The vessels were incubated for 60 min at 50°; [^{14}C]O_2 arising from the decarboxylation of [U-^{14}C]oxaloacetic acid produced by the GOT-catalyzed transamination with α-ketoglutaric acid of any enzymatically generated L-[U-^{14}C]aspartic acid was trapped in a 5-μl droplet of 40% (w/v) KOH deposited on the lid of the closed vessels, then counted as previously described.

Amylase. Amylase (EC 3.2.1.1) was measured in the supernatant (12,000 *g*, 3 min) of pancreatic homogenates by a commercially available semi-automated nephelometric technique utilizing the Perkin–Elmer Coleman 91 Amylase–Lipase Analyzer (Perkin-Elmer Corp., Oakbrook, IL).

Lactic acid dehydrogenase. Lactic acid dehydrogenase (EC 1.1.1.27) was measured in organ supernatants by a standard spectrophotometric technique in which NADH was used as the indicator.

Acid phosphatase. Acid phosphatase (EC 3.1.3.2) was measured in the supernatant of liver homogenates by a colorimetric technique utilizing, as substrate, 0.1 mM naphthol AS-BI phosphate in 0.1 M sodium acetate buffer, pH 5.0. After incubation at 37° for 60 min, the reaction mixtures were adjusted to pH 9.5 with NaOH and the absorbance was read at 420 nm.

Hepatic microsomal enzymes. Cytochrome P-450, aminopyrine *N*-demethylase and cytochrome *c* reductase (EC 1.6.99.3) were kindly assayed in hepatic microsomes by Dr. T. Gram of this institute using previously published techniques [8–11].

Measurement of L-*asparagine and* L-*glutamine*

L-Asparagine and L-glutamine were measured in aliquots of the 95° heated (10 min) 12,000 *g* supernatant of tissue homogenates, according to the procedures outlined by Cooney *et al.* [3, 12].

Protein estimation

Protein estimation was conducted utilizing the methodology of Lowry *et al.* [13] with bovine serum albumin as a standard.

Attempts to prevent or reverse the inhibition of L-*asparagine synthetase*

To determine if the ordinary substrates of L-asparagine synthetase could prevent or reverse the inhibition produced by maleimide, maleimide was added to 50 μl of crude tumoral L-asparagine synthetase to a final molarity of 0.001 M either 15 min before or 15 min after L-glutamine (10 mM), NH_4Cl (20 mM), ATP-$MgCl_2$ (1 mM), L-aspartic acid (0.01 mM), dithiothreitol (3 mM) or mercaptoethanol (3 mM). After 30 min at 25°, the enzyme was diluted 4-fold with 0.1 M Tris–HCl buffer, pH 7.4, and assayed in the usual manner.

Incorporation of L-[*U*-^{14}*C*]*valine into proteins*

Male BDF_1 mice bearing subcutaneous nodules of L5178Y/AR were treated intraperitoneally with 6 mg/kg of maleimide or with normal saline. One hr and 48 hr later, 10 μCi L-[U-^{14}C]valine was injected intraperitoneally. The liver, pancreas and tumor were removed 60 min later and each was homogenized in 1 ml of 5% perchloric acid. The homogenates were centrifuged at 12,000 *g* for 10 min, and the pellets were washed twice with 1 ml of 5% perchloric acid and twice with 1 ml of 80% ethanol, resuspended and recentrifuged at 12,000 *g* for 10 min. Excess ethanol was decanted, the pellets were air dried for 16 hr at 4°, and dissolved in 1 ml of 1 M NaOH; then radioactivity of a suitable aliquot was measured by scintillation spectrometry.

Measurement of the incorporation of [^{14}*C*]*formate into nucleic acids*

Male BDF_1 mice bearing subcutaneous nodules of L5278Y/AR were treated intraperitoneally with maleimide at a dose of 6 mg/kg. One hr and 48 hr later 10 μCi [^{14}C]formate (sp. act. 20 μCi/μmole, Mallinkrodt, St. Louis, MO) was injected intraperitoneally. One hr later the liver, tumor and pancreas were removed, washed well with saline, blotted, and homogenized in 3 ml of 5% perchloric acid. After centrifugation at 2000 *g* for 30 min, the supernatant was removed and discarded. The pellet was washed twice with 5% perchloric acid and twice with 80% ethanol, then air dried at 4° for 16 hr. Three ml of 10% (w/v) NaCl was added and nucleic acids were extracted by heating the mixtures at 95° for 20 min followed by centrifugation at 1000 *g* for 30 min. The radioactivity of a 100-μl aliquot of the resultant solution of sodium nucleates was measured by scintillation spectrometry. DNA content was measured colorimetrically [14].

Toxicologic studies

Groups of 20 male BDF_1 mice were injected intraperitoneally with 3, 6, 9 or 12 mg/kg of maleimide or intravenously with 6, 9, 12 or 15 mg/kg of maleimide (saline was the vehicle, each mouse receiving 0.01 ml/g body weight). The LD_{50} was calculated on the basis of the mortality observed 14 days after administration of the drug.

In a separate experiment, groups of 20 BDF_1 mice received either L-asparagine, 1 g/kg, or saline subcutaneously 0.5 hr before the intravenous injection of maleimide, 9 mg/kg. Deaths were recorded daily and the LD_{50} was calculated as above.

For histopathology, groups of male BDF_1 mice were injected intraperitoneally with 6 or 9 mg/kg of maleimide or intravenously with 9 or 12 mg/kg of maleimide; 48 and 72 hr later, animals were bled from the retro-orbital sinus or were sacrificed by cervical dislocation and various organs collected in buffered neutral formalin. Determinations of hemoglobin, white blood cell count, and red blood cell count of blood from individual mice and determination of blood urea nitrogen, creatinine, lactic acid dehydrogenase, alkaline phosphatase, soluble L-glutamate pyruvate transaminase, amylase, sodium, potassium and chloride in the serum pooled from 4 to 8 mice were performed using standard clinical techniques. Because of the necessity of pooling blood samples for the latter determinations, standard deviations were not available.

Routine hematoxylin and eosin stained sections of formalin-fixed tissue were reviewed by Dr. Jerrold Ward of the Laboratory of Toxicology of the National Cancer Institute.

Tissue culture

L1210 murine leukemia cells were grown in suspension culture at 37° in RPMI 1630 medium containing 20% heat-inactivated fetal calf serum, penicillin (60 μg/ml) and streptomycin (135 μg/ml). Cells, grown in tightly capped plastic tissue culture flasks (Falcon No. 3024), were inoculated at 2.5×10^5 cells/ml. Untreated cells maintained logarithmic growth during the course of the experiments with a doubling time of 11 hr.

Cells were treated with cold, freshly made solutions of maleimide prepared in 0.15 M NaCl and 5 mM HPO_4 at pH 7.4, at predetermined concentrations to achieve a 24-hr growth rate inhibition of approximately 50 and 90 per cent.

At 6 hr and 24 hr after drug treatment, 1 to 2×10^6 cell aliquots were washed with isotonic phosphate buffered saline, pH 7.4, treated for 10 min with phosphate buffered saline at one-half isotonicity, then fixed with a 3:1 (v/v) ethanol/acetic acid solution for 10 min. Cells were stained with 0.2% orcein in 6% aqueous acetic acid solution. The percentage of cells containing mitotic figures was determined by microscopic examination of 200 cells.

DNA content distribution

Aliquots of treated and untreated cells were removed at 6 and 24 hr, fixed with formalin, and Feulgen-stained with fluorescent dye, benzoflavine. The DNA content of 10^5 cells/sample was analyzed by flow microfluorometry using a modified Cytofluorograph model 4801 (Bio/Physics Systems, Inc., Mahopec, NY) interfaced with an NS-633 pulse height analyzer (Northern Scientific, Inc., Middleton, WI). The fixation and staining procedures were substantially as described elsewhere [15] but were modified by incorporating 0.5% Triton X-100 in all solutions used during the staining procedure to prevent cell clumping, and by the use of plastic tubes (Falcon No. 2057) to reduce the loss of cells by adherence to tube walls. In addition, an equal number of cells for each sample were stained, since it has been determined by our laboratory and also reported elsewhere [16] that the intensity of cell-staining increases with the increasing number of cells stained. For a constant number of cells, the amount of dye bound is proportional to the DNA content of the cell. This allows construction of a DNA frequency distribution curve of each cell population studied.

Influence of wound-washing with maleimide on local tumor recurrence after surgical removal of Lewis-Lung Carcinoma

Inasmuch as maleimide appears to be locally cytotoxic (see Results) experiments were conducted using this drug for wound-washing after surgical removal of tumor tissue, in order to examine its influence on the incidence of local tumor recurrence.

A Lewis-Lung tumor cell suspension (0.1 ml; 10^8 cells) was injected intramuscularly into the calf of the left hind leg of BDF_1 mice. Surgical amputation of the entire tumor-bearing leg was performed on day 7, 10, 13, 16 and 19 tumor inoculation. A circumferential skin incision was made around the upper thigh, as high as possible above the area of tumor growth. Care was taken to avoid spreading of tumor cells. The skin was carefully retracted and a ligature was drawn tightly around the neck of the femur above the trochanter major. The muscle and femur were cut with heavy scissors, as close to the ligature as possible. The wound was closed by pulling skin over the stump and clamping it with metal wound clips.

Groups of ten mice subjected to surgery were divided into treatment and control subgroups. Each mouse in the first subgroup received careful wound-washing of the stump and the surrounding area with 4 ml of a 0.1% (w/v) maleimide solution in isotonic saline. The wound in the control subgroup was washed with 4 ml of isotonic saline.

Cyclophosphamide (225 mg/kg) was administered intraperitoneally 6 or 3 days before the surgical treatment.

Body weight and incidence of local tumor recurrence were recorded weekly. The final evaluation of the incidence rate of local tumor recurrence was calculated 140 days after tumor inoculation.

RESULTS

Toxicologic studies with maleimide

Little data were available in the literature on the effects of maleimide *in vivo*. Particularly lacking was basic information on the LD_{50} of the compound, and on the pathologic and clinical pathologic changes produced by it. Because these considerations became important at the outset of our studies with maleimide *in vivo*, it was decided to examine the toxicity of this drug formally.

Initial studies using intraperitoneally injected maleimide in BDF_1 mice revealed a LD_{50} of 9 mg/kg. Few animals died after 48 hr (in a 14-day observation period) and most died on the day after injection. Approximately 50 per cent of the mice given the LD_{50} (or greater doses) convulsed. Hemolysis was observed 4 hr after the intraperitoneal administration of maleimide, 9 mg/kg.* As noted below, however, this hemolysis was not of sufficient degree to lower the hematocrit nor was hemoglobinuria observed. Clinical pathologic observations on animals bled 4 and 48 hr after the intraperitoneal or intravenous administration of maleimide, 9 mg/kg, are summarized in Table 1. It can be seen that no alteration in serum calcium or glucose exists to explain the convulsions observed in these mice. Not shown in the table is a lack of significant alteration in serum sodium, potassium and magnesium concentrations as well. Of note in Table 1 is the observation that hemoconcentration at 4 hr was seen only in animals injected intraperitoneally; granulocytosis appeared at 48 hr in both groups, but was more marked when the drug was given intraperitoneally. Significant azotemia also developed at 48 hr in both groups of animals receiving maleimide. No significant alteration of serum amylase, lactic acid dehydrogenase, creatine phosphokinase, alkaline phosphatase or phosphorus was observed.

Forty-eight hr after intraperitoneal administration of maleimide (9 mg/kg) to male BDF_1 mice, histopathologic examination revealed superficial necrosis and inflammation of liver, pancreas and peritoneal membranes, attesting to the highly toxic local effects of intraperitoneally administered drug. Apparently, such local effects were responsible for the granulocytosis reported above. In addition, there was depletion of hepatic glycogen and cytoplasmic vacuolization, which fat stains revealed to be partially due to lipid deposition in the hepatocyte. Focal renal cortical tubular necrosis, abundant tubular protein casts and evidence of tubular regeneration also were associated with intraperitoneal injection. The renal pelvis of one animal revealed striking epithelial proliferation. Because of their focal nature, it appeared plausible that these changes might be related to the route of administration of the drug. Additional studies, therefore, were conducted by a second parenteral route: after intravenous administration, the LD_{50} of maleimide in BDF_1 mice also was 9 mg/kg. Pretreatment with L-asparagine, 1 g/kg, i.v. 0.5 hr before maleimide, did not significantly alter mortality. Again, convulsions were observed in many recipients. Histologic observations of animals sacrificed 48 hr after intravenous injection of 9 or 12 mg/kg of maleimide revealed a complete lack of the peritonitis and superficial necrosis of liver and pancreas seen with intraperitoneal dosing. In addition, glycogen depletion and vacuolization in the liver were less striking when the intravenous route was used. Renal changes were entirely absent. In the following organs, no consistent histologic change was seen: brain, lung, heart, stomach, small intestine, spleen, adrenal, bone marrow and testis. The most likely cause of death in the intraperitoneal group is concluded to be peritonitis. Renal failure very likely also contributed to death in the highest dose group. The cause of death in the intravenously treated group is less clear, although the LD_{50} is similar. Neurotoxicity, liver toxicity and possible renal toxicity (azotemia without definite renal lesions on light microscopy) probably all contributed to death in the intravenous group.

Toxicologic studies of maleimide in tissue culture

In order to examine the toxicity of maleimide on a cellular level and because sulfhydryl reagents are known to be inhibitors of the mitotic apparatus [17], the interaction of maleimide with L1210 murine leukemia cells in tissue culture was studied next with emphasis on cytotoxicity, cytokinetics and alterations in nucleic acid content of cells exposed to the drug.

Concentrations of maleimide inhibiting growth by 45 per cent caused only slight changes in the cellular DNA distribution curve at 6 hr, indicative of some accumulation of cells in early S-phase. After 24 hr of

* Incubation of a 1:1 suspension of isotonic saline-washed mouse erythrocytes with concentrations of maleimide ranging from 8×10^{-2} M to 5×10^{-6} M provoked no hemolysis after 15 min of incubation at 25°. However, after 16 hr under the same conditions total hemolysis was seen at all concentrations above 1×10^{-5} M; 50 per cent hemolysis was produced at 1×10^{-5} M and less than 5 per cent hemolysis at 5×10^{-6} M.

Table 1. Clinical pathologic changes after the administration of maleimide to BDF_1 mice*

Test (units)	Control	Maleimide (9 mg/kg, i.p.) 4 hr	Maleimide (9 mg/kg, i.p.) 48 hr	Maleimide (9 mg/kg, i.v.) 4 hr	Maleimide (9 mg/kg, i.v.) 48 hr
Calcium (mg/100 ml)	9.0	9.0	9.2	9.0	9.3
Glucose (mg/100 ml)	208	188	139	220	178
BUN (mg/100 ml)	24		52	27	72
Creatinine (mg/100 ml)	1.4		2.9		
SGPT (I.U.)	492		415	620	496
Hemoglobin (g/100 ml)	14.1 + 0.3	19.1 ± 0.3†	16.2 + 0.7†	14.8 ± 0.2	14.1 ± 0.7
Hematocrit (ml/100 ml)	44 ± 0.8	60 ± 1.3†	51 ± 1.2†	46 ± 0.6	45 ± 2.7
WBC (number/mm^3)	8340 ± 810		6400 ± 590		7000 ± 1460
Granulocytes (number/mm^3)	1130 ± 60		4995 ± 565†		2960 ± 1580

* Mice were bled from the ophthalmic sinus at the times indicated after treatment with 9 mg/kg of maleimide (i.v. or i.p.) or saline (i.v. or i.p.) (control). Serum was pooled from four to eight mice for the chemical procedures. No standard error, therefore, is available. Heparinized blood from individual mice was used for hematologic studies. Values for the latter are means of five mice.

† $P < 0.05$.

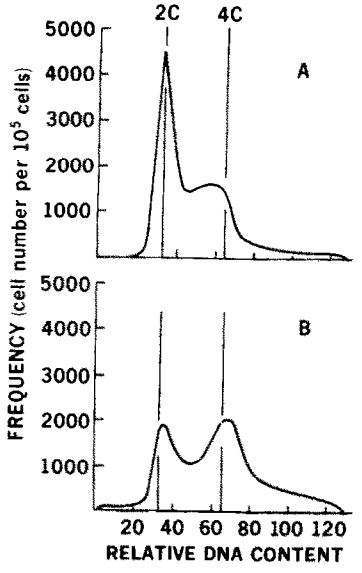


Fig. 2. Influence of maleimide on DNA distribution frequency of L1210 cells in culture. Details of the experimental techniques are given in Materials and Methods. In panel A, cells were exposed only to isotonic saline; in panel B, cells were exposed to maleimide, 1.1×10^{-5} M, for 24 hr before measurements of DNA frequency distribution. The mitotic index was not altered when compared to control, over the 24 hr of incubation with maleimide (5.6×10^{-6} M and 1.1×10^{-5} M). At 24 hr, the growth rate of L1210 cells in culture was inhibited by 45 per cent with 5.6×10^{-6} M maleimide and by 95 per cent with 1.1×10^{-5} M maleimide.

exposure, the distribution pattern had reverted essentially to that of an exponential growth pattern, indicating a general slowing of cell transit through all phases of the cell cycle.

After 6 hr of exposure to a higher concentration (which inhibited growth rate by 95 per cent at 24 hr), the cellular DNA distribution curve was essentially identical to that of the control (Fig. 2A). However, after 24 hr of such exposure, a large peak of cells with a 4C DNA content was found, indicating that cells had accumulated in (G_2 + M) phase. This signified a relative block in cell cycle transit somewhere in the G_2 phase or in mitosis (Fig. 2B). Since there was a low mitotic index at this time (Fig. 2b, legend), the relative block was concluded to have occurred in the G_2 phase. In addition, the presence after 24 hr of a peak of cells with a 2C DNA content (G_1-phase) and a considerable population of cells with DNA content between 2C and 4C (S-phase) warranted the conclusion that progression through the cell cycle had been generally retarded.

In summary, although maleimide did produce growth inhibition and a shift in the cellular DNA content distribution of L1210 cells in culture, there was no evidence in this system of inhibitory activity against the mitotic apparatus *per se*. It is relevant to point out that studies also were carried out with the hydrolysis product of maleimide, maleamic acid. No growth inhibition and no significant changes in the DNA distribution curve were observed after a 24-hr incubation of L1210 cells with 1.1×10^{-5} M maleamic acid.

Interaction of maleimide with the enzymes of L-*asparagine metabolism*

The establishment of tolerated doses of maleimide permitted the initiation of pharmacologic studies with the agent. Prior to use of maleimide *in vivo*, however, it was necessary to characterize its potency *in vitro* vs the enzymes responsible for the metabolism of L-asparagine.

Interactions of maleimide with L-*asparagine synthetase* in vitro. Maleimide was found to be a powerful inhibitor of L-asparagine synthetase of leukemia 5178Y/AR and of pancreas (Table 2). Moreover, the compound inhibited the utilization of L-glutamine to a somewhat stronger degree than the utilization of ammonia with the enzyme from both sources.

To determine whether reaction with sulfhydryl functions by maleimide was the mechanism of its inhibition of L-asparagine synthetase from L5178Y/AR, the partially purified enzyme was exposed to inhibitor in the presence or absence of its substrates and of dithiothreitol or mercaptoethanol. Saturating concentrations of ATP–$MgCl_2$, and L-glutamine or an augmented concentration of L-aspartic acid added before or after maleimide did not change the extent of inhibition observed (Table 3). However, when dithiothreitol or mercaptoethanol was added to the enzyme in advance of maleimide, the action of the inhibitor was completely prevented. Significantly less reversal was seen if maleimide was added first. It is noteworthy that dialysis against 0.1 M Tris–HCl at a pH of 8.4 failed to reverse established inhibition.

Kinetically, the inhibition exerted by maleimide appeared to be noncompetitive with ammonia, L-glutamine and L-aspartic acid as the variable substrates (Fig. 3). However, in view of the irreversibility of the inhibition, such kinetic patterns must be interpreted with caution.

Since maleimide proved to be such a potent inhibitor of L-asparagine synthetase from L5178Y/AR, a series of related compounds were examined in the hope of establishing a structure–activity relationship. In Table 4, the results of these experiments are presented.

The effect of *N*-substitution of maleimide is illustrated by compounds 1–17. In general, addition of

Table 2. Inhibition of L-asparagine synthetase of L5178Y/AR and mouse pancreas by maleimide, *in vitro**

Drug concn (mM)	% Inhibition of L-asparagine synthetase			
	L5178Y/AR		Mouse pancreas	
	Substrate		Substrate	
	L-Glutamine	NH_4Cl	L-Glutamine	NH_4Cl
0.01	0	0	0	0
0.1	18	5	0	0
1.0	92	45	84	32
10.0	94	47	94	55

* In a final volume of 15 μl were combined 5 μl of the radioactive mixture containing L-glutamine or NH_4Cl, 5 μl of the pH 7.0 drug solution to give the final concentrations listed and 5 μl of a 12,000 *g* (3 min) supernatant of a tumor or pancreas homogenate. The mixtures were assayed for L-asparagine synthetase activity as described in Methods.

Table 3. Prevention and reversal of inhibition by maleimide of L-asparagine synthetase of L5178Y/AR, *in vitro**

Counter agent	L-Asparagine synthesized (nmoles/mg protein/hr) No maleimide	Maleimide added before counter agent	% Reversal of inhibition	Maleimide added after counter agent	% Prevention of inhibition
Dithiothreitol (3 mM)	42.6	3.3	8.0	42.9	100.0
Mercaptoethanol (3 mM)	37.2	1.8	4.7	47.2	100.0
NH_4Cl (20 mM)	32.3	1.8	5.7	2.6	8.1
L-Glutamine (10 mM)	36.2	0.5	1.5	0.3	0.9
ATP-$MgCl_2$ (1 mM)	33.1	1.5	4.7	1.3	4.2
L-Aspartic acid (0.01 mM)	29.2	0	0	1.0	3.4
None (H_2O)	30.7	0.6	2.2	1.0	3.2

* Fifty μl of the 12,000 g (3 min) supernatant of L5178Y/AR homogenate rendered 1 mM in maleimide was incubated for 30 min at 37° in the presence and absence of the counter agents listed in the table. The incubation mixtures then were assayed for L-asparagine synthetase activity, as outlined in Materials and Methods.

small alkyl side chains or small cyclic groups diminishes the inhibition of L-glutamine utilization by L-asparagine synthetase, but does not drastically change the inhibition of ammonia utilization.* One notable exception to this pattern is *N*-methyl maleimide which shows no inhibitory activity against ammonia utilization. In this regard, it resembles the classical antagonists of L-glutamine, DON (6-diazo-5-oxo-L-norleucine) and CONV (5-diazo-4-oxo-L-norvaline). Parenthetically, it is worthwhile pointing out that the antitumor antibiotic Showdomycin (compound 8) has significant inhibitory activity vs both substrates.

Addition of large aromatic structures (compounds 10–16) or electron-withdrawing groups (as in compound 9) drastically reduces or eliminates inhibitory activity toward both substrates. However, conjoining of two maleimide molecules by a phenyl or alkyl bridge (compounds 19–22) results in significant inhibition. Substitution on the carbons adjoining the double bond (compounds 24 and 25) reduces inhibition, while substitution of large groups at both positions 3 and 4 (compound 23) eliminates inhibitory activity toward both substrates.

Opening the ring structure of maleimide to maleic acid or maleamic acid completely eliminates inhibitory activity even at higher concentrations. In addi-

* This trend is the opposite of that seen when *N*-substituted maleimides were examined as inhibitors of Na^+-K^+-ATPase of erythrocyte ghosts [18]. In that study, substitution at the *N*-position increased inhibitory potency proportionately to the oil/water partition coefficient of the substituent. Presumably the sensitive sulfhydryl group is contained in a lipophilic region of the cell membrane. In the case of L-asparagine synthetase, however, steric effects apparently are more important.

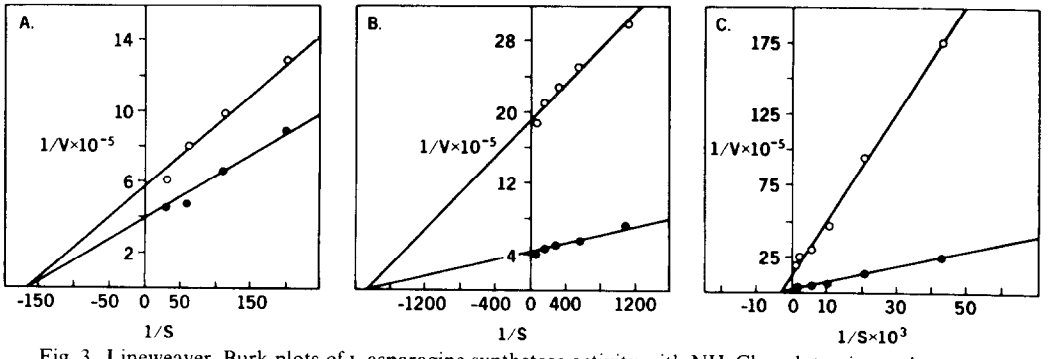


Fig. 3. Lineweaver–Burk plots of L-asparagine synthetase activity with NH_4Cl, L-glutamine and L-aspartic acid as substrates in the presence and absence of maleimide. In a final volume of 15 μl were combined (A) 5 μl of the radioactive mixture for L-asparagine synthetase activity measurement (see Materials and Methods) differing only in NH_4Cl concentrations (5–33 mM); or (B) 5 μl of the radioactive mixture for L-asparagine synthetase activity measurement differing only in the L-glutamine concentrations (0.9 to 13.3 mM); or (C) 5 μl of the radioactive mixture containing L-glutamine differing only in L-[4-^{14}C]aspartic acid concentrations (0.025 to 1.49 mM); and maleimide [1 mM; (○)] or 5 μl water (●) as well as 5 μl of the crude L-asparagine synthetase of L5178Y/AR. The mixture was assayed for L-asparagine synthetase activity as described in Materials and Methods. Key: S = molarity, and V = cpm.

Table 4. Inhibition of L-asparagine synthetase of L5178Y/AR, *in vitro**

Compound No.	Name	(mM)	Per cent inhibition	
			Substrate	
			L-Glutamine	NH_4Cl
1	Maleimide (1-*H*-pyrrole-2,5-dione)	1	94	50
2	*N*-methyl maleimide (1-*H*-pyrrole-2,5-dione, 1-methyl)	1	93	0
3	1-*H*-pyrrole-2,5-dione, 1-ethyl-	1	94	75
4	1-*H*-pyrrole-2,5-dione, 1-(1-methylethyl)-	1	16	25
5	1-*H*-pyrrole-2,5-dione, 1-cyclohexyl-	1	28	69
6	1-*H*-pyrrole-2,5-dione, 1-(phenylmethyl)-	1	51	46
7	1-*H*-pyrrole-2,5-dione, 1-phenyl-	1	43	76
8	Showdomycin (1-*H*-pyrrole-2,5-dione, 3-β-D-ribofuranosyl-)	1	61	51
9	1-*H*-pyrrole-2,5-dione, 1-(iodophenyl)-	1	7	27
10	1-*H*-pyrrole-2,5-dione, 1-(9-oxo-, 9-*H*-fluoren-2-yl)-	1	0	0
11	1-*H*-pyrrole-2,5-dione, 1-(9-phenathrenyl)-	1	0	0
12	1*H*-pyrrole-2,5-dione, 1-(3-fluoranthrenyl)-	1	0	0
13	1-*H*-pyrrole-2,5-dione, 1-(6-chrysenyl)-	1	0	0
14	1-*H*-pyrrole-2,5-dione, 1-(1-pyrenyl)-	1	0	0
15	1-*H*-pyrrole-2,5-dione, 1-(1-anthracenyl)-	1	0	0
16	1-*H*-pyrrole-2,5-dione, 1-(2-anthracenyl)-	1	0	0
17	1-*H*-pyrrole-2,5-dione, 1-(2,3-dihydro-1-*H*-1,2,4-triazole-3-yl)-	1	0	8
18	1-*H*-pyrrole-2,5-dione, 1,1'-(1,6-hexanediyl)bis-	1	0	0
19	1-*H*-pyrrole-2,5-dione, 1,1'-(1,4'-phenylene)bis-	1	27	12
20	1-*H*-pyrrole-2,5-dione, 1,1'-(1,2-phenylene)bis-	1	28	12
21	1-*H*-pyrrole-2,5-dione, 1,1'-(1,3-propanediyl)bis-	1	68	40
22	1-*H*-pyrrole-2,5-dione, 1,1'-(1,2-ethanediyl)bis-	1	11	16
23	1-*H*-pyrrole-2,5-dione, 3-ethoxy-4-phenyl-	1	0	0
24	1-*H*-pyrrole-2,5-dione, 3,4-dichloro-1-methyl-	1	31	0
25	1-*H*-pyrrole-2,5-dione, 3-bromo-1-methyl-	1	27	0
26	Maleic acid (2-butenedioic acid, (*Z*)-)	1	0	0
		10	0	0
27	2-Butenedioic acid, 2,3-dichloro-, diethylester, (*Z*)-	1	0	0
28	2-Butenedioic acid, dihydrazide, (*Z*)-	1	0	0
29	2-Butenedioic acid, 2,3-dibromo-, (*Z*)-	1	0	0
30	3,4-Dichloromaleic anhydride (2,5-furandione, 3,4-dichloro-)	1	0	0
31	Maleamic acid (2-butenoic acid, 4-amino-4-oxo-, (*Z*)-)	1	0	0
32	2-Butenoic acid, 4-oxo-4-4-(2-propenylamino)-(*Z*)-	1	0	0
33	2-Butenoic acid, 4,4'-[1,4-cyclohexanediylbis (methyleneimino]bis[4-oxo-, (*Z*, *Z*)-	1	0	0
34	2-Butenoic acid, 4-[(aminocarbonyl)amino]-4-oxo-, (*Z*)-	1	0	0
35	2-Butenoic acid, 4-amino(2-aminoanthracenyl)-4-oxo-, (*Z*)-	1	58	42
36	2-Butenoic acid, 4-amino(1-aminoanthracenyl)-4-oxo-, (*Z*)-	1	44	45
37	2-Butenoic acid, 4-(3-fluoroanthrenylamino)-4-oxo-, (*Z*)-	1	38	56
38	Succinamic acid, (butanoic acid, 4-amino-4-oxo-)	1	0	0
39	Butanoic acid, 4-amino-2,4-dioxo-	1	0	0
40	Butanoic acid, 4-amino-3-chloro-4-oxo-, (*S*)-	10	0	25
41	Butanoic acid, 4-amino-3-chloro-4-oxo-(*R*)-	1	0	0
42	Succinic acid (butanedioic acid)	10	0	0
43	Succinic acid anhydride (2,5-furandione, dihydro-)	10	0	0
44	Succinamide (Butanediamide)	10	0	0
45	Succinimide (1-*H*-pyrrole-2,5-dione, dihydro-)	10	0	0
46	1-*H*-pyrrole-2,5-dione, 1-bromo-dihydro-	1	94	100
		0.01	31	30
47	1-*H*-pyrrole-2,5-dione, 1-chloro-dihydro-	1	91	77
		0.01	14	27
48	1-*H*-pyrrole-2,5-dione, 1-hydroxy-dihydro-	1	24	0
49	1-*H*-pyrrole-2,5-dione, 1-ethyl-dihydro-	1	0	0
50	2-Propenoic acid, 3-(9-phenanthrenylamino)-, (*Z*)	1	38	45
51	2(5-*H*)-furanone, 5-hydroxy-	0.1	0	0
52	2(5-*H*)-furanone, 5-chloro-	0.1	0	0
53	2(5-*H*)-furanone, 5-hydroxy-	0.1	0	0
54	2(5-*H*)-furanone, 4-bromo-5-methoxy-	1.5	32	93
		0.15	0	27
55	2(5-*H*)-furanone, 3-chloro-5-methoxy-	1	0	43
56	2(5-*H*)-furanone, 4-chloro-5-methoxy-	1	44	67
57	2(5-*H*)-furanone, 3-bromo-5-methoxy-	1	23	42

* L-Asparagine synthetase activity was measured *in vitro* according to the assay procedure outlined in Materials and Methods, in the presence and absence of the drugs at concentrations listed in the Table.

tion, representatives of a series of derivatives of maleamic and maleic acids (compounds 26–34) as well as of maleic anhydride (compound 30) failed to show inhibition, with the unexplained exception of the anthracene substituted maleamic acids (compounds 35–37). A similar compound (compound 50) with only a 3-carbon alkenyl group does exhibit significant inhibitory activity.

Reduction of the double bond of maleimide to succinimide (compound 45) and opening of the ring structure to succinic acid, succinamic acid, or succinamide eliminate all inhibitory activity. Succinic anhydride (compound 43) is also inert. The only derivatives of these compounds (compounds 38–49) which show significant inhibitory potency are the *N*-halogenated succinimide derivatives, compounds 46 and 47. In experiments not shown, it was found that dithiothreitol, at a concentratvon of 10 mM, completely blocked the inhibitory action of *N*-bromosuccinimide (compound 46) at a concentration of 1 mM; L-histidine was inert as a counteragent.

A number of furanone analogues of maleimide also were tested; several showed prominent inhibitory activity (compounds 54–57).

Interaction of maleimide with L-asparagine synthetase, in vivo. Pursuant to the establishment of lethal and non-lethal dose schedules for maleimide in mice, and prompted by the finding that the drug was strongly inhibitory towards L-asparagine synthetase, *in vitro*, it became feasible to examine the influence of this sulfhydryl reagent on tumor and pancreatic L-asparagine synthetase, *in vivo*.

The results of such studies are presented in Table 5. It can be seen that inhibition of L-asparagine synthetase is dependent on the dose of maleimide and the time after injection of the drug. A single intraperitoneal dose of maleimide, 8 mg/kg, markedly depressed tumoral L-asparagine synthetase activity beginning 24 hr after treatment and continuing for at least 3 days. Similar results were seen in the pancreas. Pretreatment of tumor-bearing mice with neutral reduced glutathione, 500 mg/kg, subcutaneously, 30 min before an intraperitoneal dose of maleimide, 8 mg/kg, failed to alter the pattern of mortality in the recipients (4/15 fatalities in the group pretreated with glutathione vs 6/15 fatalities in the group receiving maleimide alone, at 72 hr after injection) or to counteract the degree of inhibition of L-asparagine synthetase seen in tumor or pancreas (76 and 74 per cent inhibition in tumors of mice given glutathione plus maleimide, and maleimide alone, respectively; 71 and 52 per cent inhibition in pancreas of mice given glutathione plus maleimide, and maleimide alone, respectively).

The failure of maleimide to inhibit L-asparagine synthetase soon after injection stands in contrast to its immediate action *in vitro*, and to the action *in vivo* of DON (6-diazo-5-oxo-L-norleucine), another powerful inhibitor of the enzyme [19–21]. To explain this effect it can be speculated that maleimide must be metabolized before it can act as an inhibitor of L-asparagine synthetase *in vivo*.* Alternatively, it is possible that the drug is inhibiting protein synthesis and, therefore, the biosynthesis of L-asparagine synthetase. There is precedent for such an effect in the case of *N*-ethyl maleimide which is known to interrupt protein synthesis by dissociating ribosomes [22].

However, a direct experimental examination of the incorporation of L-[U-^{14}C]valine into protein of L5178Y/AR tumor nodules, pancreas and liver revealed that the process was insignificantly affected by maleimide 48 hr after administration of 6 mg/kg intraperitoneally (Table 6), although there was a 58

* Inhibition of L-asparagine synthetase from L5178Y/AR was not found to be progressive *in vitro* when the enzyme was incubated with maleimide over a concentration range of 1×10^{-3} to 1×10^{-7} M at 4° for periods of 1–24 hr.

Table 5. Inhibition of L-asparagine synthesis by maleimide, *in vivo**

Drug concn (mg/kg × days)	Time of sacrifice (hours past last injection)	% Inhibition of L-asparagine Synthetase			
		L5178Y/AR		Pancreas	
		Substrate		Substrate	
		L-Glutamine	NH_4Cl	L-Glutamine	NH_4Cl
2 mg/kg × 1	3	0			
	24	21			
2 mg/kg × 5	4	13	7	38†	31†
4 mg/kg × 3	4	69‡	51§	64§	57§
8 mg/kg × 1	3	0	0	0	0
	4	4	28	15	4
	8	6			
	17	23			
	24	53§			
	48	79§		62†	58†
	72	93§		75†	75†

* BDF_1 mice bearing L5178Y/AR were injected intraperitoneally at the dose schedule listed in the table or with saline. At the appropriate times indicated, all mice were killed by cervical dislocation, their pancreas and/or tumor removed, homogenized, and assayed for L-asparagine synthetase activity as described in Materials and Methods.

† $P < 0.02$, compared with saline-treated controls.

‡ $P < 0.01$.

§ $P < 0.001$.

Table 6. Inhibition of the synthesis of protein and DNA by maleimide, *in vivo**

Treatment	L-[U-^{14}C]valine (pCi/μg of protein)			[^{14}C]formate (pCi/μg of DNA)		
	Tumor	Liver	Pancreas	Tumor	Liver	Pancreas
Saline	2.22 ± 0.22	2.79 ± 0.25	18.12 ± 1.72	7.95 ± 1.71	48.39 ± 6.81	11.06 ± 0.84
Maleimide (6 mg/kg) 1 hr before L-[^{14}C]valine or L-[^{14}C]formate	0.94 ± 0.06† (58)	2.26 ± 0.18 (12)	14.50 ± 1.28 (20)	4.65 ± 0.55‡ (42)	72.39 ± 10.55 (0)	11.10 ± 1.00 (0)
Maleimide 6 mg/kg) 48 hr before L-[^{14}C]valine or L-[^{14}C]formate	1.76 ± 0.22 (21)	4.29 ± 0.67 (0)	16.78 ± 1.38 (8)	4.98 ± 0.48 (38)	38.34 ± 3.10 (21)	10.32 ± 1.01 (7)

* Details of the experimental procedure are given in Materials and Methods. Numbers in parentheses denote per cent inhibition.
† $P < 0.05$.
‡ $P < 0.01$.

per cent inhibition at 1 hr in tumor only. It is noteworthy that intraperitoneal maleimide also failed to alter nucleic acid biosynthesis in pancreas, but did inhibit this process in tumor (Table 6). The reason for these disparities in organ susceptibility is unclear, especially when it is recalled that the compound produced histopathologic changes in pancreas when it was injected intraperitoneally. It is possible that the drug did in fact inhibit protein and/or DNA synthesis in the pancreatic parenchyma, but that this action was masked by the infiltration of newly formed neutrophils actively incorporating labeled valine or formate.

It is also possible that the intraperitoneal route of administration of maleimide was somehow responsible for the delayed inhibition of L-asparagine synthetase. Attempts to demonstrate inhibition of this enzyme by maleimide delivered via the intravenous route of injection failed, i.e. no inhibition whatsoever of the enzyme from tumor or pancreas was seen 1–48 hr after intravenous injection of 6 mg/kg of maleimide. In order to reach subcutaneous tumor nodules, it would appear that intraperitoneally administered drug, like intravenously administered drug, would have to be transported in the blood. To explain the puzzling influence that the route of administration has on inhibition of L-asparagine synthetase by maleimide, it could be speculated that lymphatics interconnect the peritoneum and subcutis of the groin in the tumor-bearing mouse.

The specificity of the interaction of maleimide with L-asparagine synthetase was studied in two ways: by comparing the inhibitory potency of this agent *in vivo* with that of other sulfhydryl reagents, and by comparing the action of maleimide on L-asparagine synthetase vis-a-vis its action on a panel of enzymes representative of diverse cellular compartments and functions.

In the category of inhibitors of L-asparagine synthetase, there are two classes of clinically useful agents: active-site-specific thiol alkylators, and agents with non-specific or general thiol reactivity. *In vitro*, representatives of both classes inhibit the enzyme to a roughly comparable degree: approximately 70 per cent at 1 mM. *In vivo*, however, distinctions emerge. Thus, DON and its congeners, CONV and Azotomycin [19, 23, 24], agents known to alkylate thiols at the catalytic center of L-asparagine synthetase, inhibit it immediately after parenteral injection. In the latter category, which includes ethacrynic acid [25] and Mapharsen (data not shown), negligible inhibition of murine L-asparagine synthetase follows parenteral injection, whether at early or late time periods, after doses of 25 and 50 mg/kg, for ethacrynic acid and Mapharsen respectively. Maleimide, alone of agents in the latter category, exerts powerful delayed inhibition. In this respect, then, it is unique.

In like manner, the interaction of maleimide with a panel of other representative enzymes was examined *in vivo* (Table 7). Some activities were unchanged or elevated 72 hr after treatment (as exemplified by acid phosphatase in liver and LDH in liver and pancreas). Some were modestly depressed (as exemplified by amylase in pancreas and amino-pyrine-*N*-demethylase in liver), but L-asparagine synthetase was reduced to 25 per cent of control in pancreas and 5 per cent of control in tumor, a degree of inhibition not approached by any other enzyme investigated. Thus,

Table 7. Influence of maleimide on several murine enzymes, *in vivo**

Enzyme	Tissue	Treatment		Ratio of maleimide-treated to saline-treated controls
		Maleimide (8 mg/kg)	Saline	
L-Asparagine synthetase†	Pancreas	375 ± 42‡	1501 ± 75	0.24‡
L-Asparagine synthetase†	L5178Y/AR	86 ± 14‡	1736 ± 196	0.04‡
L-Asparagine transaminase†	Liver	512 ± 66‡	274 ± 32	1.9
L-Asparaginase†	Liver	229 ± 31	216 ± 22	1.1
Amylase§	Pancreas	2.4 ± 0.5‡	4.5 ± 0.6	0.53‡
Lactic dehydrogenase	Pancreas	2.6 ± 0.2‡	0.93 ± 0.3	2.8‡
Lactic dehydrogenase‖	L5178Y/AR	6.9 ± 1.4	10.3 ± 1.6	0.67
Lactic dehydrogenase	Liver	17.0 ± 1.3‡	10.4 ± 1.3	1.6‡
Aminopyrine *N*-demethylase¶	Liver	5.23 ± 1.28‡	9.04 ± 0.74	0.58‡
Cytochrome P-450**	Liver	0.048 ± 0.006‡	0.076 ± 0.007	0.63‡
NADPH cytochrome *c* reductase¶	Liver	232 ± 20	223 ± 14	1.0
Acid phosphatase††	Liver	48 ± 4	44 ± 4	1.1
Acid phosphatase††	L5178Y/AR	20 ± 1	32 + 4	0.59

* Groups of ten BDF mice were treated with maleimide (8 mg/kg) intraperitoneally or with saline; 72 hr later the animals were killed by cervical dislocation, relevant organs or tumor collected, and enzyme assays performed on homogenates or microsomal fractions as described in Materials and Methods. L-Asparaginase and L-asparagine transaminase activities were measured under conditions in which the enzymes were undersaturated with L-asparagine and, therefore, the values reported are not indicative of the V_{max}. Shown is the mean ± S.E. of five determinations. Statistical analysis was performed with Student's *t*-test.

† Measured in nmoles/g wet weight tissue/hr.

‡ $P < 0.05$.

§ (Coleman 91 Units) × 10^4.

‖ I.U./g wet weight tissue.

¶ Measured in nmoles of product formed/mg of protein/min at 37°.

** Δ O.D./mg of protein.

†† Arbitrary O.D. units.

while L-asparagine synthetase is not uniquely sensitive to delayed inhibition by maleimide, it is unusually sensitive. In this connection, it is noteworthy that other enzymes involved in L-asparagine metabolism, L-asparagine transaminase and L-asparaginase, did not show inhibition, *in vivo* (see below).

The observation that intraperitoneally injected maleimide so powerfully inhibited L-asparagine synthetase of pancreas prompted an examination of the influence of the drug on the concentration of L-asparagine in that organ. It was found that a dose of maleimide of 3 mg/kg increased the pool of L-asparagine modestly, but significantly, whereas no such effect was demonstrable at a higher dose, 6 mg/kg (Table 8). On this basis, it seems possible that the lower dose of maleimide impeded the utilization of L-asparagine, but as has been seen, not its synthesis, whereas the higher dose curtailed the biosynthesis of the amide and its utilization to an equivalent degree, so that a normal concentration of L-asparagine was maintained. In support of this argument, it was found that cycloheximide, an inhibitor of protein synthesis, raised the free L-asparagine pool size, presumably by inhibition of utilization, whereas maleimide plus cycloheximide significantly reduced this effect at a dose of 6 mg/kg.

In order to determine whether maleimide also was capable of interfering with the pool size of L-glutamine, the next higher homologue of L-asparagine, measurements of the concentration of this amide were made after the intraperitoneal injection of the drug by several dose schedules.

Four hr after a single intraperitoneal dose of maleimide, 8 mg/kg, the pool size of L-glutamine in tumor had increased to 19.6 nmoles/mg of protein as compared to 12.2 nmoles/mg of protein in tumors of saline-treated control mice; this rise was significant ($P < 0.05$). No such rise was seen in the pancreata of tumor-bearing mice with this dose schedule. However, three daily injections of maleimide, 4 mg/kg, raised the concentration of L-glutamine from 35.7 nmoles/mg of protein in the pancreata of control mice to 55.0 nmoles/mg of protein in the pancreata of drug-treated mice and from 16.0 nmoles/mg of protein in control tumor (L5178Y/AR) to 33.0 nmoles/mg of protein in tumors of drug-treated animals. Both elevations are significant ($P < 0.05$). Alterations such as these are also produced by the prototypical antagonists of L-glutamine, DON and Azotomycin [21, 23]. Inasmuch as these latter two drugs are known to react via covalent attachment to critical sulfhydryl functions on one or more of the L-glutamine amido transferases, it is possible that maleimide is operating analogously, i.e. that it is inhibiting the enzymes of L-glutamine utilization. Inhibition of protein synthesis, utilizing L-glutamine, has been considered above.

Interaction of maleimide with mouse hepatic L-asparaginase in vitro. Since a purified mammalian hepatic L-asparaginase was unavailable, the interaction of maleimide with crude, soluble L-asparaginase of mouse liver was studied next.

Maleimide was found to inhibit this hydrolase in a dose-related manner. However, *N*-methyl, *N*-ethyl and *N*-isopropyl maleimide produced inhibition at high concentrations, but stimulation at lower molarities (Table 9). Apparently, the nature of the substitution on the ring nitrogen determines the presence or absence of this stimulatory behavior. Precedent for the activation of enzymes by thiol reagents exists [24]. At present it is unclear whether such biphasic effects of *N*-substituted maleimides will be demonstrable with pure preparations of hepatic L-asparaginase.

In order to determine whether the inhibition produced by maleimide was reversible, hepatic L-asparaginase was dialyzed exhaustively after exposure to 5 mM maleimide, a concentration shown to produce 90 per cent inhibition. No reversal was observed, even when dithiothreitol was present in the dialysis bath in molar excess. These results are compatible with typical sulfhydryl binding, and are consonant with the findings of Tower *et al.* [26] that L-asparaginase from

Table 8. Effect of maleimide on the activity of L-asparagine synthetase and on the free L-asparagine pool size in mouse pancreas*

Treatment	L-Asparagine synthesized (nmoles/g ± S. E.)	Per cent inhibition of L-asparagine synthetase	Free L-asparagine pool size (nmoles/g ± S. E.)
Saline (0.2 ml)	1464.7 ± 92.4		207.2 ± 16.8
Maleimide (3 mg/kg) followed by saline (0.2 ml)	1424.7 ± 53.3	2.7	312.8 ± 34.7†
Maleimide (6 mg/kg) followed by saline (0.2 ml)	695.5 ± 79.4†	52.5	223.5 ± 17.8
Maleimide (3 mg/kg) followed by cycloheximide (100 mg/kg)	1482.6 ± 128.1	0	649.6 ± 48.2†
Maleimide (6 mg/kg) followed by cycloheximide (100 mg/kg)	488.6 ± 87.8†,‡	66.6	450.2 ± 78.7†,‡
Cycloheximide (100 mg/kg)	1450.0 ± 230.5	0	700.5 ± 32.6†

* BDF_1 mice were injected intraperitoneally with maleimide, 3 mg/kg or 6 mg/kg, or with saline. Twenty-four hr later all food was removed. Water was given *ad lib*. Forty-eight hr after treatment, the mice were injected i.p. with cycloheximide (100 mg/kg) or saline. One and one-half hr after the last injection, all mice were killed by cervical dislocation, their pancreas removed and homogenized, and the 12,000 *g* supernatant was boiled for 10 min, and then assayed radiometrically for L-asparagine as described in Materials and Methods.

† $P < 0.05$, compared to saline-treated control animals.

‡‡ $P < 0.05$, compared to cycloheximide-treated animals.

Table 9. Effect of maleimide, *N*-methyl maleimide, *N*-ethyl maleimide and *N*-isopropyl maleimide on the activity of mouse hepatic L-asparaginase, *in vitro**

Drug concn (mM)	% Activity of hepatic L-asparaginase in the presence of: Maleimide	*N*-methyl maleimide	*N*-methyl maleimide	*N*-isopropyl maleimide
0.31	82	106	90	92
0.62	63	146	122	146
1.25	14	25	51	78
2.50	10	7	8	16
5.00	8	8	7	7
10.00	3	6	6	6

* Five μl L-[U-^{14}C]asparagine (final molarity 1.6×10^{-4} M) was incubated with 5 μl of hepatic L-asparaginase (see Materials and Methods) in the presence and absence of maleimide, *N*-methyl maleimide, *N*-ethyl maleimide and *N*-isopropyl maleimide at the concentrations listed in the table for 60 min at 25°. The reaction was terminated by heating the closed vessels at 95° for 10 min. Subsequent recovery of the resulting L-[U-^{14}C]aspartic acid was conducted as described in Materials and Methods. Given are the results of a typical experiment. While the biphasic nature of the interaction of *N*-ethyl maleimide with hepatic L-asparaginase was reproduced in at least three additional experiments, the molar concentrations producing stimulating or inhibition varied from experiment to experiment (cf. Fig. 4). For this reason, a full dose–response was advisable in each study.

guinea pig plasma is susceptible to inhibition by parachloromercuriphenylsulfonate, a powerful sulfhydryl reagent.

In order to determine whether sulfhydryl binding was the mechanism for the stimulation of hepatic L-asparaginase by *N*-methyl maleimide, *N*-ethyl maleimide and *N*-isopropyl maleimide, the influence of dithiothreitol on this phenomenon was examined. In Fig. 4 (panels A and B), it can be seen that dithiothreitol itself appears to be a weak inhibitor of L-asparaginase. In Fig. 4B, however, it can be seen that dithiothreitol reverses the stimulation of L-asparaginase produced by low concentrations of *N*-ethyl maleimide.

Interaction of maleimide with L-*asparagine transaminase* in vitro. *In vitro*, L-asparagine transaminase from mouse liver was inhibited by maleimide in a dose-related way with 25 per cent inhibition at 1.87 mM and 55 per cent inhibition at 7.5 mM. Similar inhibition was seen with *N*-ethyl maleimide. Since L-asparagine transaminase can generate L-asparagine, and since the concentration of this enzyme in mouse liver exceeds that of L-asparagine synthetase by several orders of magnitude, it has been suggested that the transaminase might play a role in the biosynthesis of L-asparagine [27]. While this suggestion is not very likely on other grounds (high concentrations of α-ketosuccinamic acid are required to demonstrate

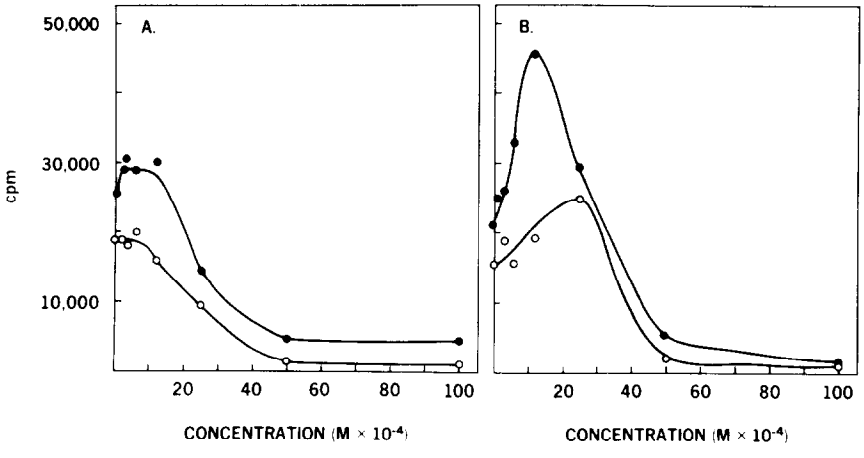


Fig. 4. Effect of dithiothreitol on the stimulation and inhibition *in vitro* of mouse hepatic L-asparaginase by maleimide and *N*-ethyl maleimide. L-Asparaginase activity in mouse liver was measured *in vitro* using L-[U-^{14}C]asparagine as described in Materials and Methods in the presence of maleimide (A) or *N*-ethyl maleimide (B) at the concentrations shown in the figure. For the preparation of the enzyme, mouse liver was homogenized 1:4 in either 0.1 M Tris–HCl buffer, pH 7.6, containing 0.5 mM disodium-EDTA (●) or 0.1 M Tris–HCl buffer, pH 7.6, containing 0.5 mM disodium-EDTA and 1 mM dithiothreitol (○), and then dialyzed at 4° for 3 hr against 1000 volumes of the homogenizing buffer.

the effect and α-ketosuccinamic acid has no known source except L-asparagine), the use of sulfhydryl reagents to inhibit L-asparagine transaminase and L-asparaginase simultaneously might offer some insight into the contributions of these enzymes to the homeostasis of L-asparagine, *in vivo*. Therefore, an examination of the influence of parenteral maleimide on the activity of L-asparaginase and L-asparagine transaminase in liver was conducted. Male BDF_1 mice were sacrificed at 3 hr and 24 hr after an intraperitoneal dose of 2 mg/kg of maleimide, 36 hr after an intraperitoneal dose of 4 mg/kg of the drug, and, as reported in Table 5, 72 hr after an intraperitoneal injection of 8 mg/kg. At none of these doses, and at none of the time periods studied, was significant inhibition *in vivo* of either L-asparaginase or L-asparagine transaminase activity produced by maleimide.

Antitumor activity

In view of the striking and protracted inhibition of L-asparagine synthetase which maleimide produces *in vivo*, and in light of the striking cytotoxicity of the agent toward cells in culture, the antitumor action of the drug was studied in mice bearing the native and L-asparaginase-resistant variant of the L5178Y leukemia. In both tumors, maleimide given intraperitoneally, 6 mg/kg, on day 8 after tumor implantation, brought about a 20–30 per cent reduction in net tumor volume. Widespread cytopathic changes in the tumor were the most salient histopathologic effects of the drug. Nevertheless, the survival time for maleimide-treated mice did not differ significantly from that of the saline-treated control animals—14.0 days vs 14.5 days. A review of data of the Division of Cancer Treatment, National Cancer Institute, revealed maleimide also to be inactive against Ehrlich ascites carcinoma, leukemia 1210, and leukemia P388 when tested in the standard screening protocol [28].

Despite this failure to demonstrate a significant therapeutic action of maleimide, it seemed possible that the drug, in its capacity as inhibitor of L-asparagine synthetase, could confer sensitivity to L-asparaginase on L5178Y AR whose resistance to L-asparaginase correlates with augmented levels of L-asparagine synthetase. Toward this end, mice bearing L5178Y AR were treated daily from day 7 after tumor implantation with L-asparaginase, 1000 I.U./kg, intraperitoneally, and with maleimide, 2 mg/kg, intraperitoneally. Control mice received no treatment, maleimide alone or L-asparaginase alone. As judged by survival times, the treatments were ineffective alone or in combination.

Influence of wound-washing with maleimide on local recurrence of the Lewis-Lung tumor

As can be seen in Table 10, wound-washing with maleimide reduced the incidence of local tumor recurrence in mice treated by surgery alone as well as in mice receiving chemotherapy plus surgery. The differences within each group were not statistically significant. However, by combining the over-all recurrence rate in mice treated by surgery and wound-washes with maleimide on days 10, 13 or 16 and in those mice treated by surgery and wound-washes on day 13 or 16 followed by cyclophosphamide (groups 2, 3 and 4, and groups 8 and 9), a statistically significant ($P < 0.01$) repression of tumor recurrence could be demonstrated in maleimide-washed animals compared with isotonic saline-washed controls (15/30 net recurrences *vs* 22/30 in groups 2, 3 and 4, and 7/69 net recurrences vs 15/69 in groups 8 and 9). Two further experiments of similar size generally confirmed the results obtained in the experiment reported here. No significant difference was observed between maleimide-washed mice and isotonic-saline-washed control animals if surgery was delayed until day 19 after tumor implantation. At this advanced stage of tumor growth, surgery was no longer radical and macroscopically visible tumor tissue remained in 9/20 animals of group 5.

DISCUSSION

The main conclusion to arise from the present studies is that maleimide, when it interacts with the mammalian enzymes of L-asparagine metabolism, *in vitro*, does so by attachment to critical sulfhydryl functions and not by virtue of any homology to the postulated cyclic anhydride of L-asparagine. Moreover, in the case of L-asparagine synthetase, at least, the target thiols do not appear to be those associated with the catalytic center, inasmuch as no protection

Table 10. Influence of wound-washing with maleimide on local tumor recurrence after removal of primary Lewis-Lung carcinoma*

Group No.	Day of surgery (after tumor implantation)	Tumor wt (g)	Cyclophosphamide (mg/kg)	Rate of tumor recurrence after wound-washing with: Saline	Maleimide
1	7	0.1		0/10	0/10
2	10	1.0		5/10	3/10
3	13	1.9		7/10	6/10
4	16	3.7		10/10	6/10
5	19	5.0		8/10	8/10
6	7	0.0	225	0/30	0/30
7	10	0.5	225	0/40	0/40
8	13	1.2	225	4/39	2/39
9	16	2.1	225	11/30	5/30
10	19	2.7	225	9/20	8/18

* Cyclophosphamide treatment and wound-washing were described in detail in Materials and Methods.

against alkylation is achieved by high concentrations of the ordinary substrates of the enzyme. In this respect, the behavior of maleimide is different from that of DON and CONV whose enzyme inhibitory action can be antagonized both by dithiothreitol and by the substrate, L-glutamine [19].

In vivo, too, maleimide differs in its behavior from other specific and non-specific sulfhydryl reagents. Thus, whereas DON and its congeners all inhibit L-asparagine synthetase immediately after their parenteral administration, maleimide requires 1–3 days to do so. In addition, whereas maleimide ultimately engenders strong inhibition of this target enzyme, *in vivo*, two other non-specific but potent thiol-reactive drugs, *in vitro*, mapharsen and ethacrynic acid, are, by comparison, feeble or inert, *in vivo*. Relevant, too, is the observation that maleimide either failed to inhibit or exerted only modest inhibition, *in vivo*, on a panel of microsomal, lysosomal, and soluble enzymes related or unrelated to L-asparagine metabolism. From the family of sulfhydryl reagents it would seem that maleimide has distinctive features as an inhibitor of L-asparagine synthetase; similarly, among other cellular enzymes—insofar as these were examined L-asparagine synthetase exhibits distinctive susceptibility to this drug.

Two hypotheses could be erected to explain this delay. First, maleimide might inhibit the synthesis of L-asparagine synthetase. This effect would have to be selective for this single protein inasmuch as the data in Table 6 document that maleimide is either an insignificant or transitory inhibitor of the incorporation of radiolabeled valine into protein. Of course, it is possible that maleimide or a metabolite of maleimide represses the synthesis of L-asparagine synthetase in the same way that the L-asparagine analogue L-aspartyl-β-methylamide does [29]. A straight chain compound is a more likely candidate for this putative action. In a companion paper, we have demonstrated that maleamic acid, the proximate decomposition product of malemide, can, in fact, function as an analogue of L-asparagine [30]. Second, the necrosis seen in the peritoneum, tumor and pancreas of mice treated with intraperitoneal maleimide might be associated with the activation of proteolytic enzymes or with the production of deleterious-free radicals which could, in turn, be responsible for the destruction of L-asparagine synthetase.

Although there is a decided lag to inhibition of L-asparagine synthetase activity after the intraperitoneal administration of maleimide, this should not eliminate its consideration for use in enhancing the oncolytic effect of L-asparaginase. Moreover, it should be stressed that maleimide, in contradistinction to many of the other potent inhibitors of L-asparagine synthetase, interrupts the utilization of L-glutamine as well as of ammonia by the enzyme. Consequently, *in vivo*, where both of these substrates are present, the inhibition effected by maleimide ought to be of greater use for preventing the emergence of tumor resistance to L-asparaginase or for converting resistant cells to the sensitive state.

It should be pointed out that Showdomycin, the riboside of maleimide, has been found not to exhibit synergistic therapeutic activity with L-asparaginase vs L5178Y [31]. The analogous failure of maleimide to effect therapeutic synergism with L-asparaginase against L5168Y/AR may reflect the subtotal nature of the inhibition exerted by the drug. Quite plainly it will be necessary to eradicate the synthesis of L-asparagine completely in order to interrupt the emergence of resistant variants. In this context it is worthwhile pointing out that several L-asparagine synthetases may exist in the cell; thus, suggestions of both a soluble and particulate enzyme occur in the literature [32]. In the present paper, we have measured mainly the soluble species. Moreover, alternate routes for the synthesis of L-asparagine are known [27, 33]. Lastly, the sequestration of tumor cells adjacent to normal cells capable of elaborating and excreting L-asparagine is a logical avenue for the sustenance of L-asparaginase-sensitive cells in the face of systemic chemotherapy with L-asparaginase. It will be necessary to interrupt every route of synthesis and transportation of L-asparagine if the resistant state is to be prevented or overcome. Maleimide itself does not achieve this end, but it is reasonable to anticipate that modifications of the molecule might give rise to better and more universal inhibitors of such processes.

Acknowledgements—The assistance of Edward Mimnaugh and Dr. T. E. Gram in the measurement of the microsomal enzymes is gratefully acknowledged.

Supportive evidence that the delayed inhibition of L-asparagine synthetase produced by maleimide might be a consequence of inhibition of protein synthesis comes from recent experiments demonstrating that an i.p. dose of actinomycin D, 400 mg/kg, produces no inhibition at 1 1/2 hr, but 75 per cent inhibition of the enzyme in pancreas at 48 hr after injection of the drug.

REFERENCES

1. F. C. Steward and J. F. Thompson, *Nature, Lond.* **169,** 739 (1952).
2. V. M. Bieulich and K. Hellman, *Hoppe-Seyler's Z. physiol. Chem.* **356,** 1325 (1975).
3. D. A. Cooney and H. A. Milman, *Biochem. J.* **129,** 953 (1972).
4. J. A. Weltman, R. P. Szaro, R. Frackelton, Jr. and R. M. Dowben, *J. biol. Chem.* **248,** 3173 (1973).
5. H. A. Milman and D. A. Cooney, *Biochem. J.* **142,** 27 (1974).
6. T. E. Gram, C. L. Litterst and E. G. Mimnaugh, *Drug Metab. Dispos.* **2,** 254 (1974).
7. D. A. Cooney, H. A. Milman and R. Truitt, *Analyt. Biochem.* **41,** 583 (1971).
8. R. Estabrook, J. Peterson, J. Baron and A. Hildebrandt, *Meth. Pharmac.* **2,** 303 (1972).
9. J. Cochin and J. Axelrod, *J. Pharmac. exp. Ther.* **125,** 105 (1959).
10. C. H. Williams and H. Kamin, *J. biol. Chem.* **237,** 587 (1962).
11. P. L. Gigon, T. E. Gram and J. R. Gillette, *Molec. Pharmac.* **5,** 109 (1969).
12. D. Cooney, R. Davis and G. VanAtta, *Analyt. Biochem.* **40,** 312 (1971).
13. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
14. K. Burton, *Methods in Enzymology* (Eds. L. Grossman and K. Moldave), Vol. XIII, pp. 163 6. Academic Press, New York (1968).

15. P. M. Kraemer, L. L. Deaven, H. A. Crissman and M. A. VanDilla, *Advances in Cell and Molecular Biology* (Ed. E. J. Dupraw), Vol. II, pp. 47–108. Academic Press, New York (1972).
16. X. Yataganas and B. D. Clarkson, *J. Histochem. Cytochem.* **22,** 651 (1974).
17. D. Mazia, *SH and Growth in Glutathione* (Eds. S. Colowick, A. Lazarow, E. Racker, D. Schwar, E. Stadman and H. Walsch), pp. 1–85. Academic Press, New York (1954).
18. H. Hayashi and J. T. Penneton, *Archs. Biochem. Biophys.* **159,** 563 (1973).
19. H. N. Jayaram, D. A. Cooney, H. A. Milman, E. R. Homan and R. J. Rosenbluth, *Biochem. Pharmac.* **25,** 1571 (1976).
20. D. A. Cooney, H. N. Jayaram, H. A. Milman, E. R. Homan, R. Pittillo, R. I. Geran, J. Ryan and R. J. Rosenbluth, *Biochem. Pharmac.* **25,** 1859 (1976).
21. R. J. Rosenbluth, D. A. Cooney, H. N. Jayaram, H. A. Milman and E. R. Homan, *Biochem. Pharmac.* **25,** 1851 (1976).
22. P. B. Moore, *J. molec. Biol.* **60,** 169 (1971).
23. D. A. Cooney, J. Yavelow, R. Zlotoff, R. Bergenstal, A. Burg, R. Morrison and R. Fleischman, *Biochem. Pharmac.* **23,** 3467 (1974).
24. D. R. Twardzik, J. Luetzeler and D. V. Ablashi, *Cancer Res.* **54,** (Pt. 1), 329 (1970).
25. H. N. Jayaram, D. A. Cooney, H. A. Milman, E. R. Homan, V. M. King and E. J. Cragoe, *Biochem. Pharmac.* **24,** 1787 (1975).
26. D. B. Tower, E. L. Peters and W. C. Curtis, *J. biol. Chem.* **238,** 983 (1970).
27. A. Meister and P. E. Fraser, *J. biol. Chem.* **210,** 37 (1954).
28. R. I. Geran, N. H. Greenberg, M. M. MacDonald, A. M. Schumacher and B. J. Abbott, *Cancer Chemother. Rep.* **3,** 1 (1972).
29. J. Uren, W. P. Summers and R. F. Handschumacher, *Cancer Res.* **34,** 2940 (1974).
30. H. A. Milman, D. A. Cooney and R. G. Cable, *Biochem. Pharmac.*, in press.
31. S. Jacobs, I. Wodinsky, C. J. Kensler and J. M. Venditti, *Cancer Chemother. Rep.* **54,** 329 (1970).
32. B. Horowitz and A. Meister, *J. biol. Chem.* **247,** 6708 (1972).
33. C. Ressler, Y. H. Giza and S. N. Nigam, *J. Am. chem. Soc.* **91,** 2766 (1969).