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Evaluation of 1-(3,4-Dichlorophenyl)-4-dimethylaminomethyl-1-nonen-3-one Hydrochloride Effect on Nucleic Acid and Protein Syntheses Using Murine Leukemia L-1210 Cells

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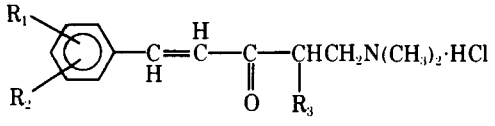
Received March 2, 1979, from the **Department of Physiological Sciences, Western College of Veterinary Medicine, and the* ‡*College of Pharmacy, University of Saskatchewan, Saskatoon, Saskatchewan, Canada, S7N 0W0.* Accepted for publication May 11, 1979.

Abstract □ Several Mannich bases derived from conjugated styryl ketones were shown to have potent cytotoxicity toward murine leukemia L-1210 cells and Walker 256 carcinosarcoma cells in culture. The most cytotoxic derivative, (*E*)-1-(3,4-dichlorophenyl)-4-dimethylaminomethyl-1-nonen-3-one hydrochloride, profoundly inhibited the incorporation of tritiated leucine into protein(s) and tritiated deoxythymidine into DNA at concentrations of 0.79–1.32 μM in L-1210 cells. At higher concentrations, incorporation of tritiated uridine into RNA and tritiated deoxyuridine into DNA was inhibited to a lesser degree. This compound failed to inhibit the enzymes thymidylate synthetase or dihydrofolate reductase up to a concentration of 10^{-4} M and was ineffective in retarding the growth of the Walker 256 carcinosarcoma in rats.

Keyphrases □ Mannich bases—derived from conjugated styryl ketones, cytotoxicity, murine leukemia L-1210 and Walker 256 carcinosarcoma cells, *in vitro* and *in vivo* □ Antineoplastic agents, potential—Mannich bases derived from conjugated styryl ketones, cytotoxicity, murine leukemia L-1210 and Walker 256 carcinosarcoma cells, *in vitro* and *in vivo* □ 1-(3,4-Dichlorophenyl)-4-dimethylaminomethyl-1-nonen-3-one hydrochloride—cytotoxicity against murine leukemia L-1210 and Walker 256 carcinosarcoma cells, *in vitro* and *in vivo*

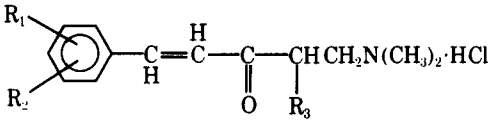
Several Mannich bases derived from acyclic α,β-unsaturated ketones (I) have shown antitumor and cytotoxic activities (1–3). Previous studies showed a correlation between cytotoxicity and murine toxicity with mitochondrial function disturbance (4). With the representative Mannich bases II*b*–II*e*, competition with coenzyme Q_{10} occurred (5). The purpose of this study was to discover whether these Mannich bases act at sites other than mitochondria or whether the biological activity observed is due solely to the effect on mitochondria.

Since series I compounds may be regarded as biological alkylating agents, a class of compounds known to interfere with the syntheses of DNA and RNA (6–8) as well as protein (9, 10), the effect on the syntheses of these biological macromolecules is reported.



I

$R_1 = R_2 = H, Cl$
$R_3 = H, CH_3, (CH_2)_4CH_3$



II

II*a*: $R_1 = R_2 = R_3 = H$
II*b*: $R_1 = R_2 = H, R_3 = (CH_2)_4CH_3$
II*c*: $R_1 = 2\text{-Cl}, R_2 = 4\text{-Cl}, R_3 = (CH_2)_4CH_3$
II*d*: $R_1 = 2\text{-Cl}, R_2 = 6\text{-Cl}, R_3 = (CH_2)_4CH_3$
II*e*: $R_1 = 3\text{-Cl}, R_2 = 4\text{-Cl}, R_3 = (CH_2)_4CH_3$

RESULTS AND DISCUSSION

Earlier work from these laboratories, which showed that the established biological alkylating agents chlorambucil and cyclophosphamide inhibited DNA synthesis, employed murine leukemia L-1210 and Walker ascites carcinoma 256 cells (11, 12). The first consideration in the present study was to establish whether representative compounds in Series I were cytotoxic to these two cell lines. Since II*a*–II*e* had previously demonstrated activity in the KB screen (4, 5), an *in vitro* screen using human epidermoid carcinoma of the nasopharynx in Eagle's medium, these compounds were examined for cytotoxicity against the L-1210 and Walker 256 cells. Both tumors showed essentially the same sensitivity to II*a*–II*e* with ID_{50} values (inhibitory doses for 50% reduction in cell numbers) in the range of 3.44–3.96 μM. The most potent compound against L-1210 cells was II*e*, with an ID_{50} of 3.46 μM (Fig. 1). This compound was used in subsequent studies.

Some reactions involved in the DNA, RNA, and protein syntheses are summarized in Scheme I. During the exponential growth phase, cells preferentially utilize deoxyuridine for the *de novo* synthesis of deoxythymidine monophosphate (13). This reaction is catalyzed by thymidylate synthetase. An alternative route for deoxythymidine monophosphate synthesis is by direct phosphorylation of deoxythymidine. Deoxythymidine monophosphate, after conversion to its corresponding triphosphate, is utilized for DNA synthesis (13). Thus, the effect of compounds on DNA synthesis can be monitored by studying the incorporation of either tritiated deoxythymidine or tritiated deoxyuridine.

Similarly, the effects of biologically active compounds on RNA synthesis can be investigated by studying their effects on the incorporation

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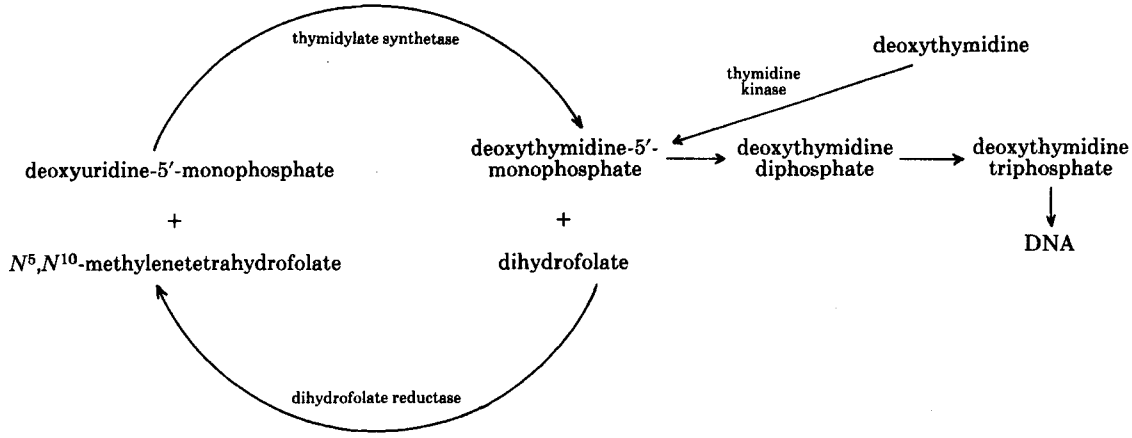

A. Metabolic pathways leading to the incorporation of deoxyuridine and deoxythymidine into DNA

$$\text{uridine} \rightarrow \text{ribonucleotides} \xrightarrow[\text{RNA polymerases}]{\text{DNA dependent}} \text{RNA}$$

B. Some reactions involved in RNA synthesis

$$\text{amino acids (}e.g.\text{, leucine)} \rightarrow \text{amino acids–}t\text{-RNA} \rightarrow\!\rightarrow \text{protein}$$

C. Some reactions involved in protein synthesis

Scheme I—Simplified schematic representation of reactions involved in the syntheses of DNA, RNA, and protein

of an appropriate labeled ribonucleoside precursor such as tritiated uridine. The ribonucleic acids are synthesized *in vivo* from four ribonucleosides, one of which is uridine. These ribonucleosides, after conversion to their corresponding triphosphates, are incorporated into RNA under the direction of a DNA template. This latter transcription process is mediated by DNA-dependent RNA polymerases (14).

Protein synthesis from amino acids is carried out by a series of complex reactions in which RNA, in its different forms, plays a major role in peptide bond formation. The present investigation studied the effect of 1-(3,4-dichlorophenyl)-4-dimethylaminomethyl-1-nonen-3-one hydrochloride (IIe) on the incorporation of tritiated leucine into protein.

The effect of IIe on the uptake of the tritiated precursors into the corresponding biological macromolecules (deoxyuridine and deoxythymidine into DNA, uridine into RNA, and leucine into protein) is summarized in Table I. Incorporation of tritiated deoxythymidine and tritiated leucine was markedly reduced, followed by tritiated uridine and tritiated deoxyuridine. Since incorporation of tritiated leucine and tritiated deoxythymidine was profoundly reduced at the dose levels of 2.64 μM (Table I), the effect of lower IIe concentrations (1.32, 0.79, and 0.26 μM) on the uptake of these precursors was investigated (Figs. 2A and 2C).

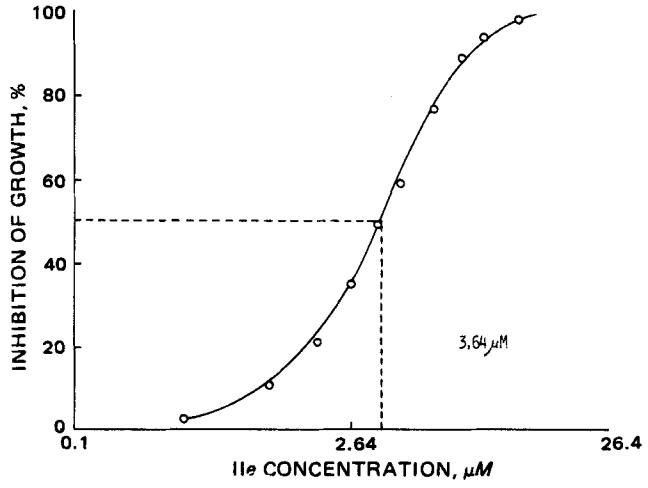

Figure 1—*Dose–response curve for IIe against L-1210 cells in tissue culture.*

The results for the IIe inhibition of tritiated uridine and tritiated deoxyuridine incorporation are shown in Figs. 2B and 2D.

After 30 min of preincubation with IIe (2.64 μM), tritiated uridine incorporation was inhibited by ~60% (Fig. 2B). Increasing either the IIe concentrations up to 13.2 μM or the incubation time up to 4 hr did not further decrease tritiated uridine incorporation into RNA. Incorporation of tritiated deoxyuridine into the DNA of drug-treated cells was inhibited only slightly (~30%) even after preincubation for 4 hr with 13.2 μM of IIe (Fig. 2D and Table I). In marked contrast, the rate of tritiated deoxythymidine incorporation into the DNA of L-1210 cells was reduced significantly (~90%) at 1.32 μM (Fig. 2C). Interestingly, the loss of tritiated deoxythymidine uptake was dependent on the IIe concentration in the medium (up to 1.32 μM) and on the incubation time. Similarly, tritiated leucine incorporation was depressed by ~60% within 10 min of preincubation in the presence of 1.32 μM of IIe (Fig. 2A).

In the presence of IIe, decreased incorporation of tritiated nucleosides into the nucleic acids could have been due to: (*a*) inactivation of the enzymes involved in DNA and RNA syntheses, (*b*) interference with the transport of nucleosides into cells, and (*c*) direct binding with the DNA template and destruction of its priming ability. Enzyme inactivation was possibly not a major factor because the inhibitory effects on the incorporation of tritiated deoxyuridine and tritiated uridine were not proportional to the IIe concentration. In addition, IIe failed to inhibit thymidylate synthetase or dihydrofolate reductase up to a concentration of 10^{-4} M (a concentration ~70-fold higher than the amount required to inhibit tritiated deoxyuridine incorporation into DNA).

Decreased incorporation of these nucleosides probably was due to interference of IIe with the nucleoside transport mechanisms. On the other hand, markedly reduced deoxythymidine incorporation obviously was due to a combination of IIe effects on tritiated deoxythymidine transport and on inactivation of enzymes (such as thymidine kinase or the thymidylate kinases) involved in the utilization of thymidine for DNA synthesis. Another possible active site of IIe may be direct binding with the DNA template, thus destroying its priming ability. The leucine incorporation rate was inhibited significantly and quickly at very low IIe concentrations. The site of action of IIe was probably interaction with some enzymes involved in protein synthesis.

These results indicate that a new biological alkylating agent, IIe, markedly inhibits DNA and protein syntheses at low concentrations, while RNA synthesis is also retarded, although to a lesser degree. Since α,β-unsaturated ketones are known to react with thiols (9, 15–18), IIe may be a general sulfhydryl alkylator which accounts, in part at least, for the observed inhibition of DNA, RNA, and protein syntheses. The effect of IIe is, however, in contrast to the established biological alkylating

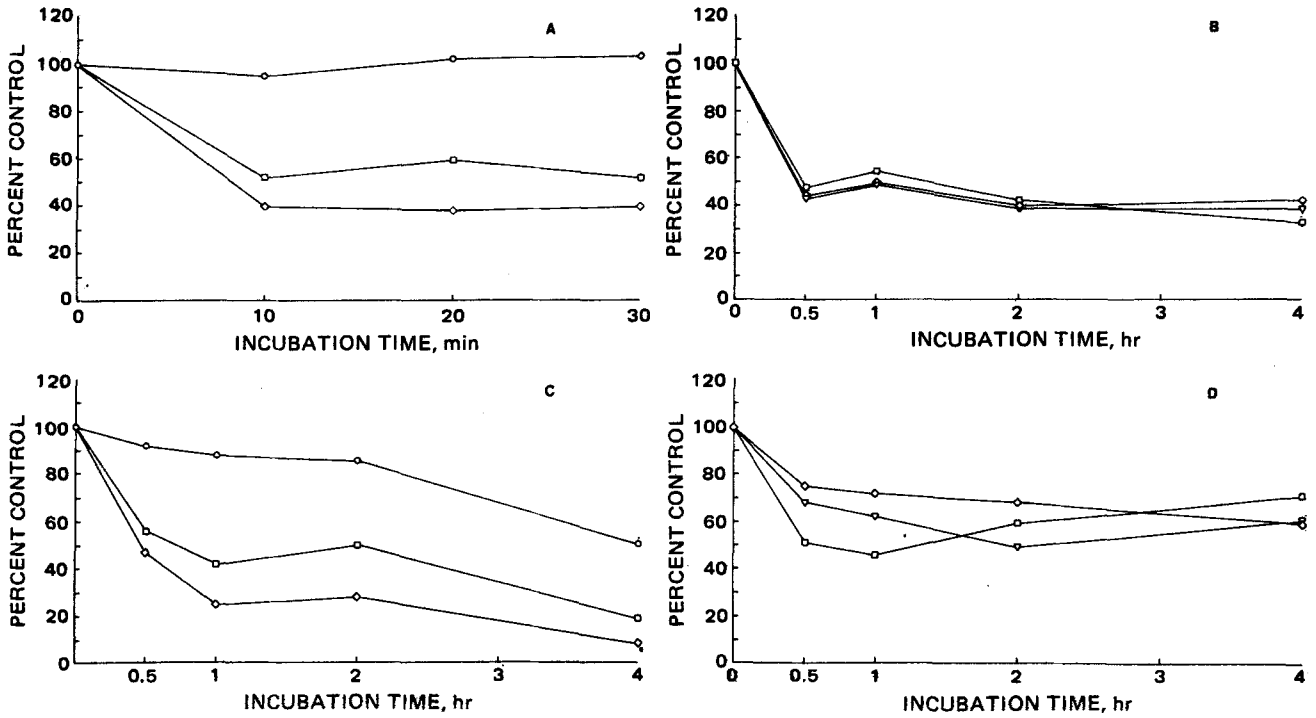

Figure 2—*Effects of IIe treatment on the incorporation of labeled precursors into macromolecules. Dose–response curves in the presence of IIe for: tritiated leucine (A) at 0.26 μM (○), 0.79 μM (□), and 1.32 μM (◇); tritiated uridine (B) at 2.64 μM (□), 7.93 μM (△), and 13.2 μM (▽); tritiated deoxythymidine (C) at 0.26 μM (○), 0.79 μM (□), and 1.32 μM (◇); and tritiated deoxyuridine (D) at 2.64 μM (◇), 7.93 μM (▽), and 13.2 μM (□).*

agents chlorambucil and cyclophosphamide, which affect DNA synthesis only at low concentrations (11, 12).

Finally, the *in vitro* studies showed that IIe along with other Mannich bases IIa–IId were cytotoxic to both L-1210 and Walker 256 cells *in vitro*. A previous report revealed that intraperitoneal administration of IIb–IIe did not produce perceptible increases in the mean survival time of mice inoculated with L-1210 leukemia (1). Therefore, it was of interest to see whether IIe would display *in vivo* activity against the Walker 256 tumors in rats, a tumor susceptible to a number of biological alkylating agents (19). Prolongation of the lifespan of animals bearing the Walker tumor did not occur at the maximum tolerated dose (25 mg/kg). At this dose, there was some evidence of toxicity, as manifested by loss of weight and diarrhea.

EXPERIMENTAL

Syntheses—The II compounds were prepared by literature procedures (1, 20).

Cell Cultures—The stock cultures of Walker ascites carcinosarcoma 256[1] and L-1210[2] cell lines were cultured in Fischers medium containing 10% horse serum[3] and were incubated at 37° in a humid carbon dioxide (5%) atmosphere. The mean generation time of the cells was determined by diluting the actively growing cells with growth medium to 1.84×10^5 cells/ml in 100-mm petri dishes. Cell counts[4] were made at 24, 48, and 72 hr. Under these conditions, the mean generation time was 16 ± 1 hr for both cell lines. Determinations were undertaken in duplicate.

Table I—Percentage Inhibition by IIe of the Incorporation of Tritiated Deoxyuridine and Tritiated Deoxythymidine into DNA, Tritiated Uridine into RNA, and Tritiated Leucine into Protein by Cultured L-1210 Cells

Dose, μM	Tritiated Deoxyuridine	Tritiated Deoxythymidine	Tritiated Uridine	Tritiated Leucine
2.64	20	84	49	71
7.93	29	90	66	78
13.2	27	86	59	79

Cell Growth Inhibition—The cytotoxicity of the II compounds toward L-1210 and Walker 256 cells was investigated as follows. Solutions of the compounds at twice the concentrations required were prepared by dissolving in water immediately prior to use, and each solution was sterilized by passage through a 45-μm filter[5]. The actively growing cells were diluted to 3.68×10^5 cells/ml with growth medium (10 ml), and the test compound solution (10 ml) was added to the cells.

The petri dishes were incubated at 37° in a 5% carbon dioxide atmosphere. The cell count[4] after 48 hr, expressed as a percentage of the control cell count, was plotted against drug concentration for 10 different concentrations of each compound. From the graph, the drug concentration giving 50% of the control cell count (ID_{50}) was obtained. All determinations were carried out in duplicate.

Effect of IIe on Incorporation of Tritiated Deoxythymidine, Tritiated Deoxyuridine, Tritiated Uridine, and Tritiated Leucine—Incorporation of the appropriate labeled precursor[6] into macromolecules was studied by a modification of the published procedure (21, 22) using L-1210 cells. A cell suspension (0.8 ml) containing $2.0–2.3 \times 10^6$ cells was placed in sterile plastic tubes, and a IIe solution was added to give the desired final concentrations. The cell suspensions were incubated at 37° in a 5% carbon dioxide atmosphere.

At regular intervals of 0.5, 1.0, 2.0, and 4.0 hr after contact with IIe, 0.1 ml of tritiated deoxythymidine (3.0 μCi, 6.52×10^{-6} *M*), tritiated deoxyuridine (3.0 μCi, 8.33×10^{-6} *M*), tritiated uridine (3.0 μCi, 5.7×10^{-6} *M*), or tritiated leucine (3.0 μCi, 2.8×10^{-6} *M*) was added to the tubes, and the samples were reincubated at 37°. In another set of experiments involving leucine incorporation, the cells were left in contact with IIe for only 10, 20, and 30 min before the tritiated leucine addition (3.0 μCi, 2.8×10^{-6} *M*). An aliquot of the cell suspension (0.2 ml from each sample) was removed at intervals of 5, 10, 15, and 20 min and added to ice-cold trichloroacetic acid (10%, 5 ml). The resultant suspension was mixed[7], left overnight at 4°, and centrifuged for 20 min at 4° at 3000 rpm.

The supernatant liquid was discarded, and the precipitate was washed three more times with cold trichloroacetic acid (10%, 5 ml) to remove unadsorbed radioactivity. The precipitate was dissolved in a solubilizer[8]

[1] Obtained from Professor J. R. Bertino, Department of Pharmacology, School of Medicine, Yale University, New Haven, Conn.
[2] Obtained from Dr. A. R. Paterson, Cancer Research Unit, University of Alberta, Edmonton, Canada.
[3] Grand Island Biological Co., New York, N.Y.
[4] Model FN Coulter counter, Coulter Electronics, Hialeah, Fla.
[5] Millipore.
[6] Amersham/Searle, Oakville, Ontario, Canada.
[7] Vortex mixer, Fisher Scientific Co., Edmonton, Alberta, Canada.
[8] NSC, Amersham/Searle, Oakville, Ontario, Canada.

(0.5 ml) and diluted with scintillation fluid[9] (12 ml), and the amount of radioactivity incorporated into DNA, RNA, or protein was counted using a liquid scintillation counter[10]. The results were expressed as the percentage of labeled precursor incorporated by treated cells divided by that of control cells. All determinations were carried out in duplicate.

Enzyme Studies—*Enzyme Isolation*—Frozen Walker 256 tumors (200 g) were cut into small cubes (1–2 cm), suspended in 500 ml of TKEM buffer (pH 7.0, 50 m*M* tromethamine hydrochloride[11] containing 100 m*M* of potassium chloride, 1 m*M* of edetate disodium, and 10 m*M* of mercaptoethanol) and homogenized for 90 sec in a blender[12]. The resulting homogenate was centrifuged at 22,000×*g*, and streptomycin sulfate solution (20%) was added dropwise with constant stirring at the ratio of 1 ml/100 ml of extract. After 30 min, the mixture was centrifuged, and the precipitate was discarded.

Solid ammonium sulfate was added to the supernatant liquid gradually with constant stirring to 35% of saturation, and the precipitate was removed by centrifugation. The ammonium sulfate concentration was raised to 55% of saturation and stirred for 30 min, and the precipitate was collected by centrifugation (Fraction I). Additional ammonium sulfate was added slowly to 95% of saturation, and the precipitate was removed by centrifugation (Fraction II). Both fractions were stored frozen at −65°. Approximately 80–90% of the total thymidylate synthetase and dihydrofolate reductase activity was recovered in Fractions I and II.

Enzyme activities were stable in the frozen state up to 3 months. A small quantity of the precipitate of Fraction I or II was dissolved in the minimum quantity of TKEM buffer, dialyzed against the same buffer for 4 hr, and centrifuged, and the supernatant liquid was used for enzyme assays.

Enzyme Activities—Thymidylate synthetase activity was assayed by a modified procedure (11) of Wahba and Friedkin (23), and dihydrofolate reductase activity was measured by the method of Perkins *et al.* (24). Reactions were initiated by the addition of substrates. Corrections were made for blanks, and all assays were carried out in duplicate.

Thymidylate Synthetase and Dihydrofolate Reductase Inhibition by IIe—An enzyme sample of Fraction I or II was incubated with varying IIe concentrations along with the assay components (except substrate) for 30 min at 22 or 37°. The reaction involving thymidylate synthetase was initiated by deoxyuridine monophosphate[10] addition, and the dihydrofolate reductase reaction was initiated with dihydrofolic acid prepared from folic acid by a literature method (25).

Effect of IIe on Growth of Walker Ascites Carcinosarcoma 256—*Tumor Transplantation*—Six-week-old male Wistar rats[13], 140–160 g, were used for all experiments. The tumor was propagated by regular transplantation every 10 days as described previously (11). The tumor size was measured on alternate days, and the tumor weight was calculated as described earlier (11).

Treatment Schedule—Fresh solutions of IIe in saline were sterilized by passage through a 45-μm filter[5] and used immediately. The concentrations were adjusted so that 0.2–0.3 ml of solution was administered at each dose. Treatment commenced on Day 8 after tumor transplantation, when the size of the tumor was 16–18 mm. Compound IIe was administered at doses of 5, 10, 15, and 25 mg/kg ip while control animals were given the same volume of solvent. Six to eight rats were used for each dose.

[9] The scintillation fluid consisted of 2,5-diphenyloxazole (5.0 g), 4-(methyl-5-phenyloxazolyl)benzene (75 mg) (obtained from Amersham/Searle, Oakville, Ontario, Canada), and scintillation grade toluene (1 liter) (obtained from Fisher Scientific Co., Edmonton, Alberta, Canada).

[10] Model 300, Isocap, Searle Analytical Inc., Chicago, Ill.

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

[12] Osteriser, Oster Corp., Milwaukee, Wis.

[13] Canadian Breeding Farm and Laboratories Ltd., Quebec, Canada.

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ACKNOWLEDGMENTS

Supported by grants from the National Cancer Institute of Canada (V. S. Gupta) and the Medical Research Council of Canada (J. R. Dimmock). Financial assistance to C. B. Nyathi was provided by the University of Saskatchewan, Connlab Holdings, Montreal, Quebec, Canada, and by the United Nations.

The authors are grateful to Walter Tsoi for technical assistance.